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A METHOD OF MEASURING THE INCREASE OR DECREASE OF THE POPULATION OF HÆMOLYTIC STREPTOCOCCI IN BLOOD.*

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IF fresh defibrinated blood infected with hæmolytic streptococci is incubated at 37° C. for two hours, and if samples of this blood, taken before and after incubation, are plated out in agar, it is generally found that fewer colonies can be cultivated from the incubated blood than from the unincubated blood. This apparent reduction in the number of living streptococci is not necessarily due to the bactericidal action of the blood, since agglutination and other influences may reduce the number of colonies which can be cultivated from the incubated blood. A true estimate of the bactericidal power is not obtained unless all the cocci originally planted in the blood are killed. A method has therefore been devised for measuring changes of streptococcal population in defibrinated blood under conditions which favour the bactericidal action of leucocytes.

Sir Almroth Wright (1918) has shown that the bactericidal power of blood for streptococci is dependent on the action of the leucocytes, and the cocci multiply freely in serum from which the cells have been removed. When a tube of defibrinated blood is infected with streptococci and incubated for some hours, the blood-cells sink comparatively rapidly to the bottom of the tube, leaving the infected serum above in which the streptococci multiply freely. Evidence of this is furnished in the following experiment:

500 c.mm. of defibrinated human blood was infected with 25 c.mm. of a broth culture of hæmolytic streptococci diluted 1 in 2000 in normal saline. The number of colonies which could be cultivated in agar from 5 c.mm. of this blood was determined. The infected blood was then incubated in a small test-tube for two hours, at the end of which time there was considerable sedimentation of the blood-cells.

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After keeping the blood at room temperature for a further period of three hours to allow more complete settlement of the cells, the supernatant serum was pipetted off and measured volumes were plated out in agar. The blood-cells from the bottom of the tube were treated in the same way. The number of colonies which grew out from the serum and cells respectively was as follows:

Number of colonies cultivated from blood before incubation .	2,580 per c.c.
,, ,, ,, supernatant serum after incubation . . .	13,600 ,,
,, ,, ,, subjacent cells after incubation	800 ,,

The streptococcal population had increased in the serum and diminished in the cells. A true indication of the bactericidal power of the blood is therefore not obtainable by incubating infected blood at rest, since the killing which takes place in the sedimented cells is counterbalanced by multiplication of cocci in the supernatant serum, and the result obtained is merely an expression of the balance between these two simultaneous and opposite processes.*

METHOD OF BRINGING THE STREPTOCOCCI AND LEUCOCYTES IN CONTACT DURING THE BACTERICIDAL TEST.

The blood which is to be tested is infected with a culture of hæmolytic streptococci suitably diluted in normal saline. The number of colonies which can be cultivated from the infected blood before incubation is determined by plating out a known volume in agar. A measured volume of 100 c.mm. of the infected blood is then placed at the bottom of a small glass capsule about 6 cm. long and 7 mm. in diameter. The neck is sealed in the blow-pipe flame, so that the blood is contained in a closed glass capsule with rounded ends. An ordinary test-tube is closed at the mouth with a rubber cap and the bottom of the test-tube is cut off. The capsule containing blood is dropped down the tube on to the rubber cap and the open end is closed with a second rubber cap. The sealed capsule is now contained in a tube which is closed at each end with a rubber cap. The tube is clamped on to the side of a pivoted square metal frame, and by means of a small electric motor the frame is rotated in the incubator at five revolutions per minute in such a way that the test-tube is inverted twice during each revolution (Fig. 1). At each inversion the

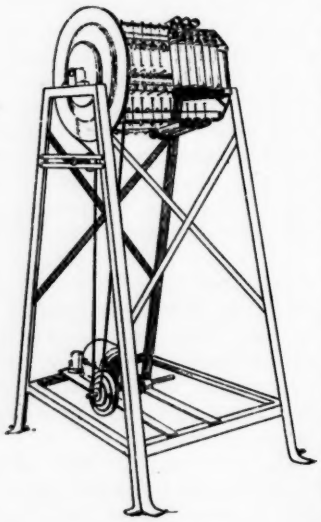

FIG. 1.

* Sedimentation of cells can be avoided to some extent by the use of slide-cells in which very thin films of blood are incubated. This is not, however, a satisfactory bactericidal method for hæmolytic streptococci, since diffuse streptococcal growth and rapidly spreading hæmolysis make it impossible to count the colonies accurately.

capsule slides from one end of the tube to the other and comes to rest on the rubber cap until the next inversion takes place. By this means the blood is kept thoroughly mixed without any frothing. After a suitable period of incubation 50 c.mm. of blood is transferred from the capsule to a few c.c. of broth in a Petri dish and after thorough mixing the dish is flooded with blood-agar. The plate is then incubated for thirty-six hours and the colonies are counted. Comparison between the number of colonies which grow out from the infected blood before incubation and the number which can be cultivated from the same volume of blood after incubation gives an indication of the bactericidal power of the blood.

Mixing machines have been used by Rosenau (1906), Fenn (1920–21), and Robertson and Sia (1924).

COMPARISON BETWEEN BACTERICIDAL POWER OF BLOOD WHEN KEPT IN MOTION AND BLOOD ALLOWED TO REMAIN AT REST.

A comparison was made between the bactericidal power of blood kept at rest in horizontal capillary pipettes and blood kept in motion on the mixing machine. For this purpose 100 c.mm. of blood infected with a suitable implant of hæmolytic streptococci was incubated at 37° C. for three hours on the mixing machine, and 50 c.mm. was then explanted into blood-agar. At the same time 50 c.mm. of the same infected blood was incubated in a stationary capillary pipette for three hours and was then explanted into blood-agar. In the following table the bactericidal actions of the same blood, tested by the two methods, are compared.

TABLE I.

Dilution of culture.	Approximate number of cocci planted in 50 c.mm. of blood.	Number of colonies which grew out from 50 c.mm. of infected blood after three hours' incubation.	
		In stationary pipettes.	On mixing machine.
1 in 25	8125	+++	55
1 in 125	1625	72	0
1 in 625	325	10	0
1 in 3125	65	3	0
1 in 15625	13	0	0

This experiment shows that blood kept in motion kills far more streptococci than blood at rest. It seems probable that this greater bactericidal effect must be due to the fact that the cocci in the moving blood are brought into more effective contact with the leucocytes. Parry Morgan (unpublished), working on phagocytosis of staphylococci, showed that the ingestion of cocci by the leucocytes was greatly increased if the microbe-blood mixture was constantly agitated during incubation. Colebrook has repeated these observations (unpublished). The method he used was to infect two parts of defibrinated blood with one part of an emulsion of staphylococci. The infected blood was then divided into two equal parts which were sealed up in glass capsules. One of the sealed capsules was incubated at rest for twelve minutes and the other

was incubated on the mixing machine for the same time. Smears were then made from each specimen and phagocytic counts gave the following results:

Blood incubated on mixing machine . 5·7 cocci per leucocyte.
„ „ at rest . . . . 1·7 „ „

Fenn (1920–21), working on phagocytosis of particles of carbon and quartz with mixtures of serum and leucocytes, found that the ingestion of particles was greatly increased by agitation of the mixtures during incubation. It is evident from these experiments that in the moving blood the cocci would be taken up in larger quantities into the leucocytes and so be prevented from multiplying in the serum.

When complete sterilization occurs it can be definitely stated that a known volume of defibrinated blood is capable of killing a known number of cocci.

Incomplete sterilization gives less definite information, since a reduction in the number of colonies which can be cultivated from a suspension of cocci after exposure to the action of defibrinated blood may result from any influence which causes aggregation of cocci. The apparent bacterial death-rate is therefore not a true index of the bactericidal power of the blood, but represents a combination of actual killing and the gathering together of living microbes into groups so that the individual cocci which grow out in close proximity are represented by one colony. Influences which are known to cause aggregation of cocci without necessarily causing their destruction are agglutination by serum, phagocytosis of cocci by leucocytes, and finally we have, especially in moving blood, to consider the clumping together of several leucocytes which may each contain living cocci. All these influences prevent even distribution of the cocci in the blood.

It is evident, therefore, that although valuable information is obtained from experiments in which incomplete sterilization occurs, yet the most critical bactericidal test is one in which complete sterilization is attained. For this reason, a method which gives an end-point in the form of complete sterilization is valuable.

INVESTIGATION OF THE NUMBER OF COLONIES WHICH CAN BE OBTAINED FROM INFECTED BLOOD AFTER VARIOUS PERIODS OF INCUBATION.

The optimum time of incubation required to secure the maximum decrease in the number of colonies which can be cultivated from the blood was investigated in the following experiments. A series of capsules containing blood, infected with suitable dilutions of a culture of *Streptococcus pyogenes*, was incubated on the mixing machine. Samples were explanted from the capsules into blood-agar after one, two and three hours' incubation. Table II gives the number of colonies which developed.

TABLE II.

Dilution of culture.	Number of colonies which developed from 50 c.mm. of infected blood before incubation.	Number of colonies which developed from 50 c.mm. of infected blood after incubation for various periods.		
		Colonies after 1 hour.	Colonies after 2 hours.	Colonies after 3 hours.
1 in 100	3600	145	82	67

A similar experiment was done to determine the number of colonies which would develop from the blood after periods of incubation longer than three hours (Table III).

TABLE III.

Dilution of culture.	Number of colonies which developed from 50 c.mm. of infected blood before incubation.	Number of colonies which developed from 50 c.mm. of infected blood after incubation for various periods.			
		Colonies after 3 hours.	Colonies after 4 hours.	Colonies after 5 hours.	Colonies after 6 hours.
1 in 100	3200	468	500	2000	+++

In these experiments the smallest number of colonies developed from the blood after about three hours' incubation, and the surviving cocci began to multiply in the blood during the fourth and fifth hours. Large numbers of streptococci were planted in the blood in order to demonstrate the multiplication of surviving cocci which begins after the bactericidal power of the blood has been exhausted. If smaller numbers of cocci are used as in an experiment quoted earlier in this paper (Table I), complete sterilization of the blood is obtained within three hours. Colebrook (unpublished) has shown that when small numbers of streptococci survive in the blood after three hours' incubation on the mixing machine, a further incubation of the blood for twenty-four hours will generally secure complete sterilization. On the other hand, when large numbers of cocci survive after three hours, further incubation leads to an enormous increase in their numbers. There is considerable variation between different strains of streptococci, but three hours is a convenient and satisfactory period for general use.

CONCLUSIONS.

1. Bactericidal tests with defibrinated blood and hæmolytic streptococci conducted in stationary pipettes are unsatisfactory owing to sedimentation of the blood-cells and consequent unchecked multiplication of the cocci in the supernatant serum.

2. Continued movement of the defibrinated blood during incubation results in a very great increase in the bactericidal effect exerted.

3. This increase in bactericidal action is associated with a greatly increased intake of streptococci on the part of the leucocytes.

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THE SEPARATION OF VIRULENT CULTURES OF *B. DIPHTHERIÆ* INTO VIRULENT AND AVIRULENT TYPES.

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In the routine production of diphtheria toxin in large quantities, there are times in these laboratories, as in others engaged in the same task, when a culture of *B. diphtheriæ* contains no toxin and this for no apparent reason. Variations in media without doubt have an influence on the quantity of toxin produced in cultures of this organism, but these would not appear to be the only factors. The question of variation of the organisms themselves is one much discussed and difficult to settle. Is the non-virulent *B. diphtheriæ*, as found naturally, a variant of the virulent type, and might this variation in a stock culture help to explain the lack of toxin production when using such cultures?

There has been some evidence that a non-virulent, non-toxic type of *B. diphtheriæ* does develop from virulent cultures of this organism. Baerthlein (1913) described three types of colonies of *B. diphtheriæ* grown from old cultures, one of which was non-virulent and non-toxic, one highly virulent and toxic and one intermediate. However, it is not clear from the fermentation reactions of the non-virulent organisms that they were still true *B. diphtheriæ*.

Bernhardt (1915), working with the "Park 8" strain, found atypical colonies of coccoid bacilli growing from old cultures. These were non-toxic and non-virulent, and on repeated subculture these properties were retained, but the morphology of the organism became typical of *B. diphtheriæ*.

Crowell (1926) picked 8 single cells from a parent single cell culture of typical virulent *B. diphtheriæ* and found the cultures from one of these cells non-virulent and non-toxic. He also found this loss of virulence to be permanent, although the morphology of the organisms was that of true *B. diphtheriæ*.

As Ledingham (1925) has stated, "it is difficult to conceive that it" (the non-virulent *B. diphtheriæ*) "is not a natural variant of the virulent form on the analogy of non-virulent forms in other bacterial groups." For instance, it has been shown that in the colon-typhoid group (Arkwright, 1921), in the bacillus of rabbit septicæmia (de Kruif, 1921), in the pneumococcus (Griffith, 1922), and in the streptococcus (Cowan, 1922), there could be isolated from single strains organisms which differed markedly in cultural and other characteristics. It was decided, therefore, to see whether some such findings could be obtained in strains of *B. diphtheriæ*, and if so whether this might throw some light on the periodic failure of production of toxin in routine culture of this bacillus.

DIFFERENTIATION OF TYPES.

The stock culture of *B. diphtheriæ*, Park 8, which had recently given trouble in respect to lack of toxin production, was plated on serum agar. After incubation for 24 hours there was little variation in the colonies. Numerous single colonies were picked and plated on serum agar. On some of these plates appeared colonies in 24 hours, which seemed rather more granular and uneven in outline than the majority. Some of the least granular and some of the most granular colonies were replated; again the most and least granular colonies were replated. This was repeated almost every day. At the end of 3 months two distinct types of colonies had developed, which were constant on subculture.

CULTURAL CHARACTERISTICS AND MORPHOLOGY OF THE TYPES DIFFERENTIATED.

One type of colony was small, raised, dense, granular, and had a slightly irregular outline. This was designated the "R" (rough) type, as it was in characteristics of appearance somewhat like the "R" type of streptococcus described by Cowan (1922). In contrast, the larger, less raised, less dense, less granular round colony with even outline was termed the "S" (smooth) type.

The "R" type, when grown in broth, grew somewhat more slowly and tended to form a poorer pellicle than the "S" type, more of the growth being down the side and in the bottom of the tube.

The individual organisms of the "R" type were fairly uniformly short, stout, barred forms, staining heavily and many lying in parallel formation. However, there were some club-shaped and irregular forms of bacilli. The "S" type showed individual forms which were thinner, longer rods with lighter staining and polar bodies. Here, too, were some irregular forms.

The differentiation of these two types from the Park 8 strain of *B. diphtheriæ* was successfully repeated, and another stock strain (89) was also found to yield the two types, but only after replating of colonies for a year (the platings could not be done daily, and little time could be spent in the examination of the colonies, so that probably this is rather an unfair estimate of average time for the process).

It was noted that in the case of both these strains, in the development of a pure "R" type, the organisms passed through a stage when the colonies were, for the most part, very large and irregular in outline and very coarsely granular, while the individual bacilli were also very large and of a great variety of shapes, and showed numerous branched forms. It was thought at first that this was probably as great a differentiation in morphology as would be found, but the picking of colonies was continued because there was still more variation in colonies and organisms than was expected if a pure type had been isolated. In this stage, too, the organisms were still virulent.

FERMENTATION TESTS.

The two types of each strain, Park 8 and 89, were grown on serum agar, subcultured into dextrose, maltose and saccharose serum water, and incubated

for 7 days. This was repeated 3 times, and in every case the result was as seen in the following table:

	Dextrose.	Maltose.	Saccharose.
Park 8 "R"	+	+	—
" "S"	+	+	—
Park 89 "R"	+	+	—
" "S"	+	+	—

The only difference in the activity of the two types was that in three of the tests 48 hours elapsed before the "R" type fermented the dextrose and maltose, whereas the "S" type did so in 24 hours.

VIRULENCE.

The "R" and "S" types of the two strains were grown on slopes of Loeffler's medium for 24 hours. Fairly heavy suspensions of each, of approximately the same density, were made in saline. 0·1 c.c. of each of these suspensions and a like amount of a known virulent *B. diphtheriæ* was injected into the skin of a guinea-pig.

Five hours later the guinea-pig was given 800 units of diphtheria antitoxin intracardially and control injections intradermally of the same suspensions as had been given earlier, *i. e.* an intradermal virulence test was made with the "R" and "S" types.

In 24 hours a small hæmorrhagic area surrounded by an area of redness was present at the site of the first injection of the control virulent culture and of both the "S" types. There was slight redness around the points of injection of the "R" types and of the second (control) injections. In 48 hours this latter redness had disappeared, but the hæmorrhagic areas at the point of injection of the "S" types and the virulent culture had increased. The guinea-pig was examined daily for a week but no further reactions developed.

That is, the "R" types, from the two strains of *B. diphtheriæ*, were non-virulent, and the "S" types from the same strains were virulent, according to skin tests for virulence. The tests were repeated with the same result.

In order to confirm or refute this finding, cultures of the "R" types were again made on slopes of Loeffler's media. Slopes of each strain having heavy growth were chosen, and the growth from each slope emulsified in about 2 c.c. of saline. One guinea-pig was given intraperitoneally the growth from one slope of the "R" type of strain Park 8; a second guinea-pig was given in the same way the growth of the same organisms from two slopes. This was repeated with the "R" type of strain 89.

All the guinea-pigs remained in good condition and were alive and well at the end of two months. It would appear, therefore, that the "R" type of *B. diphtheriæ* is non-virulent for guinea-pigs even when given in large doses.

TOXIN PRODUCTION.

Cultures of the "R" types of the two strains of *B. diptheriæ* were made in broth, from a batch of media which had been used successfully for the production of diphtheria toxin. After growing for 10 days the cultures were filtered and the filtrates tested for sterility. These proving sterile, 0·1 c.c. of

undiluted filtrate from each culture was injected intradermally into a guinea-pig to see if any toxin was present.

There was no reaction at the point of injection of these filtrates from the growth of the "R" types.

Using the "R" type of Park 8, cultures were again made in both large and small amounts of broth and incubated for 10 days. The filtrates showed no toxin reaction on intradermal injection into guinea-pigs although the growth had been good.

Each of 8 guinea-pigs was then given 5 c.c. of this filtrate subcutaneously. These showed no harmful effects and were alive and well at the end of a month.

ATTEMPTS TO IMMUNIZE GUINEA-PIGS WITH THE "R" TYPE.

It seemed rather unlikely, if the "R" type of *B. diphtheriæ* produced no toxin, that it would be of value as an antigen in the production of immunity against the virulent *B. diphtheriæ*. To obtain evidence in respect to this point, each of 6 guinea-pigs was given intraperitoneally the 24-hour growth from one slope of Loeffler's medium, of the "R" type of strain Park 8. One guinea-pig died within 24 hours, showing no apparent reddening of the suprarenals or hæmorrhagic exudate around the point of injection or in the peritoneal cavity. From its blood was cultured an organism which appeared to be the same as the one injected.

One month later each of the 5 surviving guinea-pigs was given a similar dose of the same organism intraperitoneally. All remained alive and well.

Three weeks after the second injection a Schick test was done on each of the guinea-pigs. The test was positive in every case.

At the end of another week the test was repeated, and was again positive on all of the guinea-pigs.

Forty-eight hours after this second Schick test, each of the 5 guinea-pigs and each of 6 normal control guinea-pigs was given intraperitoneally about one-third of a 24-hour growth from one slope of Loeffler's medium of an ordinary culture of the strain Park 8.

In 24 hours the 11 guinea-pigs were dead.

Each showed red suprarenals and hæmorrhagic exudate about the site of injection and into the peritoneal cavity.

Each of a second series of 6 guinea-pigs was given subcutaneously 5 c.c. of the filtrate of a 10-day culture of the "R" type of strain Park 8.

One month later a Schick test was done on 2 of the guinea-pigs. These tests were both positive. This test was repeated on these 2 guinea-pigs each week for 3 more weeks with the same result.

A month after the first dose of filtrate, each of the 4 remaining guinea-pigs was given subcutaneously another 5 c.c. of the filtrate.

One month later one of these guinea-pigs was given a Schick test. It was positive, and was again positive when repeated a month later.

One month after the second dose of filtrate the 3 remaining guinea-pigs which had not had a Schick test were each given a third injection of 5 c.c. of the filtrate subcutaneously.

A month after the third dose a Schick test was done on these 3 guinea-pigs and was positive in all.

There was no evidence in any of these experiments that the whole culture from solid media of the "R" type of *B. diphtheriæ* or the filtrate of broth cultures of this organism were of use as an antigen to produce immunity against the virulent organisms of the same strain.

The result is in accord with Ledingham's (1925) finding that the immunization of guinea-pigs with non-virulent *B. diphtheriæ* obtained from the human throat gives no protection against virulent *B. diphtheriæ*.

It was thought that the injection of the whole culture of this type might have produced some types of antibodies, although it did not confer immunity on the animals inoculated with it. Consequently a small amount of blood was taken from the ears of the guinea-pigs which had been treated with whole cultures the day before the lethal dose of the virulent culture was given to them intraperitoneally.

Complement-fixation tests were done on these sera, using the virulent culture of Park 8 as antigen. None showed any fixation.

DISCUSSION AND CONCLUSION.

The number of strains of *B. diphtheriæ* in which two types of organisms termed "R" and "S" have been differentiated is very small, namely two only, but these were the only strains worked with in this way for any considerable time. It would seem possible that the differentiation could be repeated in other strains provided a proper length of time were devoted to the separation of the two types.

There was some indication that the types kept in stock culture tended to vary somewhat in cultural and morphological characteristics but not in virulence. This, however, was not more than an indication.

It is possible that the apparent lack of virulence and toxin production by the "R" type of the strain Park 8, known to be virulent and a good toxin-producer as a rule, may be related to the occasional failure of this strain to produce toxin. It is a question whether the "R" type might overgrow, for some reason, the toxin-producing organisms. When these experiments were almost completed there was again difficulty in the routine production of diphtheria toxin with the strain Park 8.

A subculture on a serum agar plate was made from one of the culture flasks where the 10 days' growth of Park 8 had produced toxin too weak to be of use. Only one colony grew on this plate, and it was small, very dense and granular, having very much the appearance of the colonies of the "R" type which had been isolated. Part of the colony was picked into broth and part rubbed over the plate again and re-incubated. The colonies were the same as the original colony and the growth in broth was practically all in the bottom of the tube.

Dextrose, maltose and saccharose serum water tubes were inoculated with this organism and incubated for 7 days. The dextrose and maltose were fermented in 48 hours and the saccharose not in 7 days.

An intradermal skin test on a guinea-pig for virulence showed the organism

to be non-virulent, while a control test made with a fresh culture from Park 8 on the same guinea-pig was markedly virulent. The 24-hour growth from two slopes of Loeffler's medium was given to a guinea-pig intraperitoneally. The guinea-pig remained alive and well and showed no immunity at the end of 2 months.

Without further attempts to grow the "R" type in various media, it would not be fair to say that it is impossible for this type to produce toxin or for the filtrate from a 10-day culture to produce immunity. Also we cannot be certain that the whole cultures of this type might not produce some antibodies if given in large amounts and over a long period.

However, the indications from the experiments recorded are all in the same direction; from a virulent culture of *B. diphtheriæ* can be separated an avirulent, non-toxin-producing type which ferments dextrose and maltose but not saccharose, and which, from its resemblance in cultural and morphological characteristics and lack of virulence to the "R" type of streptococci, has been designated the "R" type of *B. diphtheriæ*.

I wish to thank Miss Maclennan for performing the complement-fixation tests, and the various members of the staff of the Connaught Antitoxin Laboratories for giving the benefit of their experience with *B. diphtheriæ*.

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THE *RÔLE* OF THE RETICULO-ENDOTHELIAL SYSTEM OF THE CUTIS IN EXPERIMENTAL VACCINIA AND OTHER INFECTIONS: EXPERIMENTS WITH INDIAN INK.

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In a recent brief communication (Ledingham, 1926[1]) reference was made to the general outcome of certain experiments designed to test whether the response to an intradermal inoculation of vaccinia in the rabbit could be modified in degree or character by the simultaneous injection of substances like indian ink, or by superposition of the virus on dermal sites previously infiltrated with these substances. The ulterior object of these experiments was to obtain further evidence in support of the views previously propounded by the writer (Ledingham, 1924, 1926[2]) as the result of experimental work on vaccinia in the rabbit and the histological analysis of the lesions produced. These results, both experimental and histological, lent strong support to the view that the primary attack of the vaccine virus is on the reticulo-endothelial system and not on the epidermal elements, as most of the older writers and some contemporary workers, notably Levaditi and Nicolau (1923[1,2]), have alleged.

The question of the precise nature of the attack of certain so-called filtrable viruses on skin elements is not one that lends itself to easy solution in view of the close apposition in this region of elements of epiblastic and mesoblastic origin. In natural smallpox and in experimental skin vaccinia induced by intravenous injection of the virus in the rabbit (Calmette-Guérin experiment) the virus reaches the skin by the vascular system, and the first evidence of its presence is afforded by an inflammatory lesion compounded of cellular and fluid exudate and deranged epithelia in the vicinity. The matter may be further complicated by morphological changes or appearances in epidermal cells (or corneal epithelia), suggesting at first sight the result of some sort of specific attack of virus on epithelia (*Guarnieri bodies*, *Molluscum bodies*, etc.). I have elsewhere (Ledingham, 1926[2]) had occasion to discuss the probable nature of these bodies, and I do not propose to take up this problem here.

Evidence obtained from histological analysis of the vaccinial response in dermis, subcutaneous tissue, and organs still more remote from epidermis, has shown that the reticulo-endothelial tissue of these parts may alone bear the brunt of the attack and that adjacent epidermal elements may entirely escape. Even in the brain of the rabbit, in that fatal encephalitis which is produced only by direct intracerebral inoculation of the virus, histological analysis reveals an intense disturbance of the local reticulo-endothelial system with pronounced perivascular sheathing, but without any specific disturbance of

neuroblastic elements. It is thus difficult, in vaccinia at least and in experimental herpes, to understand how, in view of the intense and early reaction of the reticulo-endothelial system to the presence of these viruses, epidermal or neuroblastic elements should be the first elements affected and the vascular changes only secondary. Moreover, when one reflects that in diseases like typhoid fever, cerebro-spinal fever or glanders, the skin exanthem reveals to histological examination masses of typhoid bacilli or meningococci associated with the dermal response, one does not infer that the infective agents of these diseases are essentially epidermotropic. The general predilection for skin must depend on other factors, and among these must be the peculiar anatomical relationships of the peripheral vascular system in this region. To these and similar vascular peculiarities elsewhere, *e. g.* in the testicles, I have alluded in a previous paper. The predilection for skin may, in fact, be correlated with its relatively small capillary area, and what Krogh (1922) describes as the "low metabolic level" prevailing there.

THE EMPLOYMENT OF "BLOCKADE" METHODS.

The injection of substances such as indian ink and various dyes for which the cells of the reticulo-endothelial system have a peculiar affinity has been employed in recent years with a view to engaging the phagocytic activity of these cells, and thereby, temporarily at any rate, to depress the response of this system to antigen parenterally introduced. It would appear on the whole, though the evidence is somewhat conflicting and contradictory, that antibody response may be modified in such conditions. Further, it has been alleged that in animals highly immunized against certain infections, or even in animals naturally immune to such, flooding of the system with indian ink and the like may cause a temporary fall in general immunity. The evidence from individual papers on this subject need not be detailed, as it does not primarily concern the particular experiments to be described here. Orientating summaries of the general results obtained will be found in recent papers and reviews by Kritschewski and Meersohn (1926) and Sacks (1926).

In the majority of the experiments dealing with "blockade" of the reticulo-endothelial system the altered state has been effected by flooding of the system with the particulate matter by intravenous injection oft repeated and frequently in combination with splenectomy. In the experiments I now detail the object was to ascertain the influence of the particulate matter when injected into the local site of proliferation of the virus—the dermis in the case of vaccinia. It was not expected that flooding with indian ink by the intravenous route would modify the cutaneous or intracutaneous response to vaccinia, but the first experiment performed was of this nature. It will be entered first as it shows that, while flooding is ineffective in modifying the dermal or cutaneous reaction, the former, at least, is altered very remarkably by the simultaneous injection of ink and virus. The ink used throughout was Gurr's preparation for spirochæte detection. It was diluted in various concentrations with physiological saline and boiled before use. The description of the protocols of various illustrative experiments will be followed by the histological findings.

TO DETERMINE THE INFLUENCE OF INK IN MIXTURE WITH VIRUS ADMINISTERED CUTANEOUSLY AND INTRACUTANEOUSLY.

Exp. (A).—Rabbit prepared by intravenous injection of Indian ink on three successive days (on each day 4 c.c. of a 1 in 32 dilution of ink). On the 4th day it was tested both cutaneously (scarification) and intracutaneously with rabbit passage lymph (diluted 1 in 40 with saline).

Result:

	Days after inoculation.					Remarks
	3	4	5	6	8	
Virus + ink (cutaneously) . .	0	+	++	++	++	Papules discrete.
„ + saline (cutaneously) .	0	+	+++	+++	+++	„ confluent.
„ + ink (intracutaneously) .	0	Pale flabby swelling.				No acute inflammatory reaction.
„ + saline (intracutaneously)	+++	+++	+++	+++	+++	Typical vaccinial response.

Summary.—The above experiment indicates that while blockading with ink by the intravenous route does not prevent cutaneous or intracutaneous takes, these, and particularly the latter, are either greatly modified in character or abolished when the lymph is inoculated in mixture with ink.

Exp. (B).—Ink concentration varying, virus constant. Unprepared rabbit as control to *Exp.* (A). Six intracutaneous and two cutaneous inoculations.

Result:

	Days after inoculation.						Remarks.
	3	4	5	6	8	10	
Virus + ink (1 in 32) .	0	+	±	±	±	+	Pale central node. No redness.
„ + „ (1 in 128) .	±	+	±	±	±	+	„ „ „
„ + „ (1 in 512) .	+	+	+	+	+	++	Larger node. No redness.
„ + „ (1 in 1024) .	+	+	+++	+++	+++	+++	Late but typical vaccinial reaction.
„ + saline . .	++	+++	+++	+++	+++	+++	Typical vaccinia.
„ + „ . .	++	+++	+++	+++	+++	+++	„ „
„ + ink (scarified) .	0	++	++	++	++	++	Discrete papules.
„ + saline (scarified)	0	+++	+++	+++	+++	+++	Confluent papules.

Summary.—With virus containing concentrations of ink from 1 in 32 to 1 in 512, the resulting intracutaneous lesions are represented by pale nodes with blackish centres, the nodes very gradually increasing in size as the ink concentration diminishes, but without any obvious acute inflammatory change. With ink in concentration of 1 in 1024, the lesion was of the typical order and indistinguishable from that of the controls. The results following cutaneous inoculation (scarification) do not materially differ, except that the tendency is for the resultant papules on the ink site to be discrete and not confluent as on the control (saline) site. This latter result may be due simply to some agglutinative action of the ink on the virus particles. As will be noted later, the mere rubbing in of ink along with virus on a scarified surface affords little histological evidence of any deep penetration of ink into subjacent dermis.

Exp. (C).—Dose of virus varying, ink concentration constant. Along one side of back was made a series of intracutaneous inoculations with ink and varying doses of virus. Along the other side was a similar series with saline and varying doses of virus.

Result:

	Days after inoculation. 3	6	9	Remarks.
Virus (1 in 2) + ink (1 in 25)	±	±	±	Pale flaccid node.
„ (1 in 10) + „	±	±	±	„ „ „
„ (1 in 100) + „	0	0	0	—
„ (1 in 1000) + „	0	0	0	—
„ (1 in 10,000) + „	—	—	—	
Virus (1 in 2) + saline	+++	++++	+++	Typical vaccinial reaction.
„ (1 in 10) + „	++	+++	+++	„ „ „
„ (1 in 100 + „	±	±	±	—
„ (1 in 1000 + saline	0	+	±	—
„ (1 in 10,000) + „	0	0	0	—

Summary.—The sample of virus (rabbit passage lymph) employed on this occasion proved of low potency by intracutaneous inoculation, but the comparative results with the higher doses of virus in the two series are very striking. No characteristic inflammatory reaction occurred when ink was present, the response being represented simply by a pale flaccid node with black centre. When the animal was killed on the 9th day it was noted that the axillary gland on the control side was, as usual, large and hæmorrhagic, while on the ink side the corresponding gland was small and pale. Cross-sections of the ink sites (in preparation for histological examination) showed simply central triangular ink masses.

HAS THE INK *PER SE* A LETHAL ACTION ON VIRUS?

In explanation of the entire change in the character of the intracutaneous response to vaccinia given in admixture with ink, the possibility of a lethal action of the ink on the virus before or after the inoculation into the dermis had in the first instance to be considered. It is well known that certain viruses, and especially vaccinia, are capable of being adsorbed by substances like animal charcoal, kieselguhr, etc. Whether death of the virus occurs after adsorption would appear to depend most probably on the nature of the adsorbent, but the available data on this point are meagre. Levaditi and Nicolau (1923) state that animal charcoal, which had adsorbed neuro-vaccine by contact with the latter for 20 hours in the cold and was finally centrifuged down, was quite active. If, however, the charcoal deposit with adsorbed virus was preserved dry at laboratory temperature, it lost its potency after 44 hours. With rabies virus the charcoal deposit always failed to infect, indicating destruction of the adsorbed virus. They state also (in footnote) that finely precipitated metallic bismuth, tantalum and sulphate of thorium not only adsorb the virus, but destroy it. In some experiments with pulverized filter material (kieselguhr and porcelain) Coplans (1926) found that the former rendered the vaccine virus inert, while the latter, in addition to its adsorbing property, concentrated the virus upon itself in an active state.

It did not appear likely that, under the conditions of experiment employed in this work, the Indian ink would exercise any destructive action on the virus, but it seemed desirable to test the point. The following are the protocols of experiments carried out with indian ink and with a sample of vegetable charcoal, "Norit" brand.

Exp. 1.—Rabbit passage lymph, after mortaring with sand and centrifuging, was added in equal parts to an ink suspension (1 in 8). As control the same lymph was mixed with equal parts of saline. The mixtures were placed in the cold at 2° C. for 48 hours. As this particular brand of ink cannot be separated by the centrifuge from the excipient, destruction of virus (if any) could be determined only by titration. Both mixtures therefore were titrated by scarification on the shaved back of the rabbit, 0·02 c.c. of each dilution being rubbed on a circular scarified area. The results on the 5th day revealed no differences in potency between the two series, each yielding typical papules in dilution of 1 in 1000, but not in higher dilutions. Contact with ink had therefore not impaired the potency of the virus.

Exp. 2.—0·25 gm. of charcoal, "Norit" brand, was added to 5 c.c. of a 1 in 10 dilution of rabbit passage lymph. After half-an-hour at room temperature the mixture was centrifuged, giving a perfectly water-clear supernatant. It was stirred and placed in the cold (2° C.) for a further 24 hours. It was then centrifuged and both supernatant and deposits tested by the scarification method. The supernatant was also tested intracutaneously in parallel with the saline-lymph control. Final reading of results on the 7th day showed large numbers of discrete papules on the area scarified with the ink deposit, and confluent papules on the control. The supernatant fluid gave entirely negative results both by scarification and by intracutaneous inoculation. It was clear, therefore, that while the charcoal had completely adsorbed the virus, the latter had not been rendered inert in the process.

SUPERPOSITION OF VIRUS ON INK SITES.

Though no evidence was obtained of any direct lethal action of ink on virus before injection of the mixture into the animal, it was considered of interest to exclude this possibility still further by determining the influence of ink already *in situ* in cutis on virus subsequently superposed intracutaneously. I append the protocols of three experiments of this nature.

Exp. 1: Superposition of Virus after 5 Hours.

Similar volumes of ink of varying concentration (1 in 16 to 1 in 2048) were inoculated intracutaneously, and 5 hours later a constant amount of virus was superposed on these ink sites. The controls were intracutaneous inoculations of virus superposed on sites infiltrated 5 hours before with saline.

Result:

	Days after inoculation.				Remarks.
	2	3	5	6	
Virus superimposed on ink (1 in 16)	0	0	±	±	Slight central node only.
" " " (1 in 32)	0	0	0	±	
" " " (1 in 64)	0	0	0	0	
" " " (1 in 128)	0	0	0	0	
" " " (1 in 256)	0	0	±	+	
" " " (1 in 512)	±	+	+	+	Typical vaccinial responses.
" " " (1 in 1024)	+	+	++	++	
" " " (1 in 2048)	+	+	+++	+++	
" " on saline	+	+++	++++	++++	
" " "	+	+++	++++	++++	
" " "	±	++	+++	++++	

Summary.—It is clear that in the spots containing the high concentrations of ink, the superposed virus failed to elicit the typical response, which supervened only when the ink concentration in the site fell to 1 in 512 or 1 in 1024. *It was noted that outside one ink spot (ink 1 in 16) there was a very definite vaccinial response, indicating that an escape of virus occurred beyond the site of deposition of the ink.*

Exp. 2: Superposition of Virus on Ink Spots at Later Intervals, 24 Hours to 49 Days.

A rabbit was inoculated intracutaneously with ink (1 in 16) on the following dates: 25 . 3, 9 . 4, 29 . 4, 3 . 5, 6 . 5, 10 . 5, 12 . 5. On 13 . 5, when an equal volume of virus was superposed on these ink areas, their ages were 49, 34, 14, 10, 7, 3 and 1 days. Two fresh spots were also inoculated at the same time. The condition of the ink spots before superposition of the virus was as follows: 49-day and 34-day spots were perfectly flat and flaccid. 14-day, 10-day and 7-day spots still showed small palpable central nodes, while 3-day and 1-day spots were still a little swollen and œdematous.

Result:

	Days after inoculation.				Remarks.
	1	2	5	7	
49-day spot	0	±	0	0	See text.
34-day spot	0	±	+	±	
14-day spot	0	±	0	0	
10-day spot	0	+	+	+	
7-day spot	0	+	+	+	
3-day spot	±	+	++	++	
1-day spot	±	+	++	++	
Fresh control spot	+	+++	++++	+++	
" " "	+	+++	++++	+++	

The appearances of the spots at various dates after inoculation of virus are given in detail.

24 hours.—49-, 34-, 14-, 10- and 7-day spots are quite pale; 3-day and 1-day spots are swollen and pinkish; fresh control spots are diffusely swollen and red.

2 days.—49-, 34- and 14-day spots show merest flabby thickening but no redness. 10-, 7-, 3- and 1-day spots show distinct flabby thickening, which is due in all probability to the progressing ink reaction (still only 9, 5, 3 days old respectively), but near the centre of each spot, at the periphery of the tattoo-mark, is a small area of reddish tint. The controls show diffuse hard swelling and redness.

5 days.—On the 5th day a more definite reading was possible. 49-day spot showed no change. 34-day spot showed a minute reddish thickened vallum in centre, later developing into a minute ulcer. 14-day spot was unchanged. 10-, 7-, 3- and 1-day spots show ascending degrees of central thickening with minute reddish ulcerating points indicating vaccinial effects.

Little further change had taken place when the animal was killed on the 7th day. So far as the macroscopic readings could tell, it appeared that inoculation of virus into the ink sites was followed by completely negative or

postponed abortive reactions, the latter being reduced to the smallest dimensions in the centre of the tattoo-mark.

At autopsy the skin bearing these spots was reflected and examined by transmitted light. Behind the control spots were many points of hæmorrhagic necrosis in the course of the vessels. There were none such at the bases of the ink spots.

Exp. 3: Further Results of Superposition of Virus.

The rabbit employed for this experiment had been thus prepared: Spots available for superposition:—*No. 1*: Ink spot, 35 days old, now quite flat and flaccid. *No. 2:* Spot into which *S. erysipelatis* had been inoculated intradermally 35 days before. *No. 3:* Spot into which *S. erysipelatis* and ink had been inoculated 35 days before. *No. 4* and *No. 5:* Ink spots 20 days old which still presented minute palpable central knobs. *No. 6:* Ink spot 2 days old.

Into these spots a similar volume of virus was inoculated and also into two fresh skin areas (*C and C'*). Further controls were (A) virus followed after 30 minutes by ink, and (B) ink followed after 30 minutes by virus.

The first definitive reading was taken on the 4th day with following result: Nos. 1, 2, 3, 4, 5, no change; No. 6, typical vaccinial response; *C* and *C'*, typical response; *A*, typical response; *B*, typical response.

Summary.—As in the previous experiment, no response took place in the 35-day-old and 20-day-old spots whether ink or *S. erysipelatis* had been the material injected initially. In the 2-day spot, however, it was evident that conditions were not unfavourable to the propagation of the virus. The response appeared later than in the controls, and it seems almost certain that escape of virus occurred beyond the region pervaded by the ink. Also in this experiment the superposition of lymph on ink after 30 minutes and that of ink on lymph after a similar interval did not give conditions unfavourable to vaccinial response, but in these cases also the naked-eye appearances pointed to escape of virus from the inhibitory influence of the ink area. Doubtless also in those experiments in which ink and virus are given simultaneously or superposed on each other at brief intervals, some balance between the ink concentration and the dose or virulence of the virus must be a determining condition of successful inhibition of vaccinial response.

The remarkable failure of the virus to cause any naked-eye response whatever when superposed on the older ink spots and healed erysipelatoid lesions, raises problems to which some answer may be forthcoming from the histological analysis of the skin lesions in the various experiments detailed here and to be discussed presently.

ALLERGIC RESULTS IN INK SPOTS.

The animal employed for the last experiment was again used after the lapse of one month for an experiment of this nature.

Four spots were taken with the following history:—*No. 1:* Ink on May 18th and June 24th. *No. 2:* Virus followed by ink on June 24th. *No. 3:* Ink followed by virus on June 24th. *No. 4:* Ink only on June 24th.

Into these spots (on July 23rd) a 1 in 50 dilution of glycerinated lymph was inoculated intracutaneously. Before inoculation the condition of the spots was as follows: Nos. 1 and 4 were flat and flaccid. When injection was made into Nos. 2 and 3, a little blood escaped at the point of injection on withdrawing the needle. This event, of great rarity in performing intracutaneous inoculations, was almost certainly due to interference with the vascularizing tissue still remaining after the first inoculation of ink and virus. Previous work had shown that even after 30 days following an inoculation of vaccinia the cutis was not completely reconstructed.

Result of experiment.—All four spots remained unchanged when virus was superposed, whereas freshly-shaved areas of skin reacted in allergic fashion to intradermal injection of virus. It would seem, then, that in the immune or partially immune animal allergic phenomena cannot be elicited by injection of virus into areas which have been previously stimulated, whether by ink and lymph or by ink alone.

EXPERIMENTS WITH *STREPTOCOCCUS ERYSIPELATIS*, DIPHTHERIA TOXIN AND ANTHRAX.

These experiments have been so far only of a preliminary character, so that I may very briefly state the results.

S. erysipelatis.—It is known that strains of streptococci from human erysipelas lesions may produce on intracutaneous injection in the rabbit spreading lesions strongly resembling erysipelas.

Exp. A.—A rabbit received two intracutaneous inoculations of *S. erysipelatis* alone, and in conjunction with ink.

Result.—Streptococcus alone: In 24 hours the site of injection was pink and swollen with a whitish core, and extending considerably beyond the circle of injection was a spreading erysipelatoid fiery lesion, with here and there whitish patches on it. At 7 days the spreading lesion was thick and hard with here and there purulent foci. At 17 days the erysipelatoid lesion had a shining granulating appearance with some cicatricial bands in it.

Streptococcus and ink.—In 24 hours the circle of injection was only faintly pink and there was no spreading erysipelas-like lesion. Even at 7 days the site of injection showed only slight swelling, and there was no trace of extension beyond the site.

Apparently, therefore, the simultaneous injection of ink prevented completely the spreading erysipelas-like effect, and confined the response strictly to the *locus* of injection.

B. anthracis.—Simultaneous injection of ink with *B. anthracis* both intra- and subcutaneously failed to prevent fatal infection in guinea-pigs.

Diphtheria bacilli and diphtheria toxin.—Experiments with these were kindly made for me by Dr. M. M. Barratt. With diphtheria bacilli inoculated intra- and subcutaneously in guinea-pigs, no modification of virulence effect was obtained by adding ink to the mixture. With diphtheria toxin, however, some striking results were obtained. The following are protocols of two experiments concerned:

Exp. A.—Two guinea-pigs received each 0·01 c.c. of diphtheria toxin (1 M.L.D.) subcutaneously. *Result:* Both dead in 2–3 days.

Two animals received the same dose in admixture with ink. *Result:* Both animals lived. One was killed for histological examination on the 11th day and the other on the 21st day. The local reaction produced by the toxin-ink mixture was intense. Over the site of injection the integument was puffy and œdematous, later becoming hard and board-like and finally sloughing off.

Exp. B.—This experiment was an exact repetition of the last, the two controls dying in 3–4 days, while the animals inoculated subcutaneously with the ink-toxin mixture recovered after severe local reaction with great loss of weight. In one month the local lesion had completely disappeared and the weight had commenced to rise.

HISTOLOGICAL ANALYSIS.

No attempt to interpret the findings detailed in this paper can be made without a detailed knowledge of the lesions produced in the dermis by ink alone. To procure suitable material for study a rabbit received on February 17th six intracutaneous injections of ink (1 in 16). At periods thereafter of 24 hours, 3 days, 6 days, 8 days, 13 days and 36 days a spot was surgically removed and immediately placed in fixative (formol-sublimate-iodine). The stain employed was, in the main, Twort's light green-neutral red mixture.

The chief histological features at the various dates were as follows:

24 hours.—Ink lying loosely in layers in centre of dermis, but more densely over vascular area overlying skin-muscle. Polynuclears have permeated the ink mass, especially in neighbourhood of vascular area, the vessels being crammed with these cells and active diapedesis is proceeding. Macrophages are present in moderate numbers in area of dermis bounding the ink mass, and some have penetrated to centre of ink mass along with polynuclears. No phagocytosis of ink at this stage by macrophages. The polynuclears at no stage exhibit phagocytic powers for ink.

3 days.—Polynuclears permeating ink mass are undergoing comminution of chromatin. Elsewhere, especially at periphery of ink mass, they are becoming the prey of macrophages. The outlying particles are being phagocyted actively by macrophages, which have now greatly increased in number. This increase has followed the stimulus given to proliferation of the resting cells lining the collagen fibres, by the opening-up of the collagen spaces with the ink-saline suspension. Under the stimulus these cells become prominent and induced to divide precisely like the corneal corpuscles in vaccinial keratitis. They are actively phagocyting ink-particles. The vessels passing up through mid-dermis from the vascular area overlying skin-muscle already show commencing proliferation of cells in their adventitial sheaths.

6 days.—The ink mass is now permeated with macrophages actively phagocyting ink while the polynuclears are reduced to clusters of comminuted chromatin. In the papillary region and in mid-dermis there is a great increase of reticulo-endothelial tissue which acts as a boundary zone to the main ink mass. The outlying ink particles are found included in mononuclears at some distance from the main mass.

8 days.—The reticulo-endothelial response progresses. Overlying skin-muscle and in apposition with the main ink mass, a new embryonic vascular area is being hollowed out to deal with the foreign matter in its vicinity.

13 days.—Main mass of ink is now collected (agglutinated) into oval masses while the peripheral particles are mostly included in macrophages. Perivascular sheathing is now very prominent in the area overlying skin-muscle and throughout dermis generally. Even between the papillæ the cellular content of the space is greatly augmented.

36 days.—The normal configuration of the dermis is almost restored. Residual ink is lying passively in oval masses in the collagen spaces. Much of it, however, is lying in macrophages at the periphery, and these phagocytes are to be found for a considerable distance away along the upper surface of the skin-muscle.

Summary of events.—The essential effect of the ink inoculation on dermis elements has been to stimulate the local reticulo-endothelial system so that the ink may be dealt with by the proliferated adventitial cells of the vessel-sheaths and the more mobile macrophages. Incidentally the proliferation of these cells is much in excess of that required to deal with ink particles that find themselves detached from the main mass, and it is most probably this diffuse proliferation of reticulo-endothelial tissue which plays the chief *rôle* in determining the negative or abortive responses to vaccinia, particularly in the superposition experiments. What function is served by the initial polynuclear response is obscure. These cells do not phagocyte ink, and in a few days disappear by chromatolysis or chromatic comminution, possibly the result of an oxidizing action on the part of the adjacent ink.

The response of the dermis to ink differs from that to vaccinia in this important particular—that there is no initial toxic action on vascular endothelium followed by congestion, hæmorrhage and focal necrosis of vascular walls. After an injection of ink the reticulo-endothelial system is directly stimulated, and at an early stage without any preliminary depression. After an inoculation of virus, on the other hand, the reparative processes on the part of the stimulated reticulo-endothelial system follow the initial toxic or inflammatory stage.

HISTOLOGY OF LESIONS PRODUCED BY INK AND VIRUS SIMULTANEOUSLY INOCULATED.

Material examined:

(*A.*) *Spot* (*virus* + *ink, 1 in 32*).—Naked-eye response *nil.* Killed 10th day.

Chief features.—Enormous hyperplasia of reticulo-endothelial tissue, especially that overlying skin-muscle. No remains of necrotic foci in mid-dermis as in (*B*) (below) and in control spot of virus only. The only evidence of attack of virus is afforded by a small spot in papillary region, where the nuclei of proliferated adventitial cells are all reduced to comminuted particles of bizarre shapes. The exudate from this small area has, in its way to the surface to form a small scab, overrun and destroyed the adjacent Malpighian layer.

(*B.*) *Spot* (*virus* + *ink, 1 in 1024*).—Naked-eye response +++ (same animal).

The histology confirms the naked-eye finding. There are typical foci of vascular necrosis and hæmorrhage with fibrinous œdema.

(*C.*) *Spot* (*ink + virus, 1 in 10*).—Naked-eye response *nil.* Killed 9th day.

There is no evidence of residual lesions that can be attributed to vaccinia. Mid-dermis shows diffuse hyperplasia of reticulo-endothelial tissue.

(*D.*) *Spot* (*ink + virus, 1 in 1000*).—Naked-eye response *nil* (same animal).

Though there was no visible response macroscopically, histological examination reveals a small spot in which the perifollicular adventitial tissue has broken down in the depth of the dermis. There is no implication of epidermis.

(*E.*) *Control spot* (*boiled virus with ink*).—Animal killed 11th day.

The chief feature is the colossal hyperplasia of the reticulo-endothelial tissue throughout the whole dermis.

(*F.*) *Control spot* (*boiled virus only*).—Animal killed 3rd day.

Even after three days there is fairly extensive hyperplasia of reticulo-endothelial tissue. The finding is important in the light of experiments in which the virus was superposed, not on ink spots, but on spots into which *S. erysipelatis* had been injected many days previously with resultant abeyance of vaccinial response. The suggestion is strong that the increased local defence is due, in part at least, to the presence of an already mobilized reticulo-endothelial hyperplasia, which may be produced, not only by ink, but also by streptococci and other substances. Histological examination of spots removed from rabbits which had responded in erysipelatoid fashion to intradermal injection of *S. erysipelatis* had revealed a very extensive spreading hyperplasia of reticulo-endothelial tissue.

HISTOLOGY OF LESIONS PRODUCED BY SUPERPOSITION OF VIRUS ON INK.

Superposition of Virus on Ink after 5 hours.

(*A.*) *Spot* (*ink, 1 in 32*).—Naked-eye response *nil.* Killed 8th day.

Histological examination revealed evidence of vaccinial attack on R.E. cells in papillary region with comminution of nuclei and disruption of cell. A small surface scab has formed and the underlying epithelial layers have been overwhelmed. It would seem that comminution of R.E. cells is the cardinal symptom of vaccinial attack, if any such takes place when the virus is incorporated with ink or superposed.

(*B.*) *Spot* (*ink, 1 in 2048*).—Naked-eye response +++ (same animal).

Here the lesion is purely vaccinial and typical. Ink in this concentration has offered no inhibition to proliferation of virus.

Superposition of Virus at Late Periods.

(*A.*) *Spot* (*superposition of virus on 49-day ink*).—Naked-eye response *nil.* Animal killed 7th day.

The section shows in the centre an oval abscess-like area surrounded by amorphous ink masses and phagocytes containing ink. The virus has caused a fresh chemotaxis of polynuclears which have penetrated into the centre of the "abscess" area. It would seem that the virus on injection has found itself among a mass of ink phagocytes on which it has exerted a necrotic action, with

setting free of their contained ink. The remaining intact ink phagocytes have formed a complete barrier to the injected virus and rendered it inert.

(*B.*) *Spot* (*superposition of virus on 10-day ink*).—Naked-eye response ++. Small swelling confined to centre of spot with minute ulcer and reddish, thickened edge. Animal killed 7th day.

There is enormous reticulo-endothelial hyperplasia extending from skin-muscle to epidermis. The ink has been only partially dealt with by macrophages, and there are great tracts of reticulo-endothelial tissue free of phagocytes. The superposition of virus has caused in its vicinity a comminution of the local proliferated tissue. There is no evidence of œdema or congestion which would indicate response on the part of the general circulation. The virus appears to attack directly the proliferated R.E. tissue in its neighbourhood.

DISCUSSION.

The experiments I have detailed show that the macroscopic dermal response to the vaccine virus may be abolished or completely modified in character and degree when the virus is injected in conjunction with an ink suspension, or superposed on a site previously infiltrated with such suspension. In the latter case the inhibition of response is obtained with greater regularity and completeness, and, indeed, it would appear that within the limits of the experiments, the inhibition is more or less directly proportional to the age of the ink site on which the virus is superposed. There is evidence also that on ground which has been infiltrated by other materials than ink, *e. g. S. erysipelatis*, a superposed vaccine virus fails to take. By inhibition of response is understood the failure of the virus to produce what is usually regarded as a typical naked-eye reaction, consisting of a raised red swelling at the site of the intradermal injection. Where such response has been annulled or modified in character, histological examination has revealed evidence of a purely focal attack on mobilized reticulo-endothelial elements with consequent cell-necrosis (disruption of cell with pyknosis and comminution of chromatin). By these elements is meant the perivascular adventitial cells and the more diffusely distributed collagen corpuscles which have in response to ink proliferated massively and widely throughout the area of inoculation. Great numbers of these proliferated cells are engaged in the phagocytosis of ink with which they may be in contact, but the response of the local R.E. system to the stimulus of ink has far exceeded that required by the tissue or actually used to deal with ink particles.

What conditions does the virus meet when superposed on an ink site? The chief vascular area overlying skin-muscle is covered at least in its central portion with the main mass of injected ink, but separated therefrom by a broad band of hyperplastic R.E. tissue and phagocytes containing ink. Spreading out radially for some distance along the skin-muscle are extensions of this hyperplastic tissue with many phagocytes. In the mid dermis surrounding the main mass of ink and extending up to the papillary region are similar collections of R.E. tissue, great tracts of which, as I have pointed out, contain cells which are not functioning as phagocytes of ink.

When virus is introduced into mid-dermis it is precluded by the main mass of ink from direct contact with the underlying vascular area—a circumstance which probably explains the absence of any marked leucotaxis, congestion and œdema. Escape of virus during injection beyond the ink area has usually led, as we have seen, to the characteristic raised red nodes of dermal vaccinia. The specific attack of the virus is thus by its very environment directed against the hyperplastic R.E. tissue among which it finds itself.

We may assume, then, that phagocytosis of virus by adjacent R.E. cells takes place. How far the R.E. cells may be capable of dealing with such phagocyted virus without detriment to themselves we have no information, beyond the fact that histologically no lesion attributable to vaccinia may be found. We have evidence, however, that necrosis of proliferated R.E. cells takes place, and may indeed be the only obvious sign of attack of virus on dermal tissue. Such disturbances may give no naked-eye evidence whatever of their presence.

It is evident, therefore, that in the inhibition of response to vaccinia under the conditions of the superposition experiments, physical or mechanical factors play a part in preventing or retarding a general vascular response with leucotaxis and œdema, but the chief *rôle* is played by the mobilized R.E. tissue which the stimulus of ink has caused to proliferate.

When the virus is injected in conjunction with ink, the inhibition of response would appear to depend on similar factors. The main vascular area is again protected from direct action of virus owing to its being adsorbed on ink (provided the ink is in sufficiently high concentration), and hence may be explained the absence of œdema and congestion. The additional stimulus to the R.E. system which the ink has contributed will heighten the normal defence against the virus. Further, in this case we have to assume the simultaneous ingestion by the macrophage of ink and of virus which has been adsorbed thereon. It is conceivable that virus so presented to the phagocyte is unable to initiate any mutual interaction and remains simply inert, the cell receptors being otherwise engaged. Jelin (1926) has suggested some such sequence of events in attempting to explain why in a particular experiment, in which a rabbit had been "blockaded" by the intravenous and intraperitoneal routes, a following injection of *B. typhosus* into the peritoneum failed to produce a fatal result. In his view the local R.E. cells, being fully engaged with particulate matter, are unable to disintegrate any phagocyted bacilli and so set free their toxin, on which, in his opinion, the essential typhoid syndrome depends. Such a theory based on lack of suitable receptors, or previous engagement of otherwise suitable receptors, obtains considerable prominence in "blockade" literature, but cannot be pursued further here in the absence of relevant knowledge. Acceptable interpretation of "blockade" phenomena must await the further multiplication of experiment. So far as the failure of response in the superposition experiments is concerned, there may possibly, in addition to the factors already observed, be another factor of a similar order to that involved in the non-excitability of skin to light stimuli for some time following a primary reaction. Such has been described in the absence of pigment.

SUMMARY AND CONCLUSIONS.

(1) The intracutaneous response to vaccinia in the rabbit is entirely modified in degree and character or completely abolished if the virus is inoculated in conjunction with indian ink of sufficient concentration.

(2) If a dermal site is infiltrated with ink, and virus is subsequently superposed on the same site, no naked-eye response may follow.

(3) Indications have been obtained that infiltration of dermis with other material, *e. g. S. erysipelatis*, may also modify the reaction to a subsequently superposed virus.

(4) The mechanism of the inhibition is discussed, and particular stress laid on the action of ink in stimulating the local reticulo-endothelial system to proliferate, and thus enhancing the defence forces of the part.

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ON SOME OF THE UNEXPLAINED DIFFICULTIES MET WITH IN CULTIVATING THE GONOCOCCUS: THE PART PLAYED BY THE AMINO-ACIDS.

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THE continual introduction of new media for the growth of the gonococcus suggests that the principles underlying the cultivation of this micro-organism are ill-understood. In our experience it has been difficult to repeat the particularly good results obtained on their special media by various authors, *e. g.* Thomson (1917) or Vedder (1915).

After experiment we have adopted a medium which we have found to be very constant and superior in our hands to those mentioned; but it has seemed to us more useful to analyse the value of the different components of this medium than to compare it with other empirical media already recommended.

The medium which we have found most satisfactory has a basis of meat

extract, and contains 1% of peptone (Parke-Davis), 0·5% of NaCl, 2% of agar and 10% of defibrinated rabbits' blood. The blood is added to the medium while fluid at 45°C., and the whole is heated subsequently for 15 minutes at 75°C. The reaction aimed at has been pH 7·6. The meat extract has been prepared by gently heating a litre of water containing 500 gm. of chopped lean meat till boiling and then leaving it boiling slowly for two hours, and finally filtering through filter-paper.

By processes of elimination, *i. e.* the use of media defective in one or more constituents or in which a substitute has been used for one constituent, it has been shown: (1) that meat extract is essential, since 1% peptone agar with 0·5% NaCl and 10% of blood heated at 75° C. either fails to grow the gonococcus or grows it very poorly; (2) that increase or decrease in the concentration of the peptone may stimulate growth or inhibit it according to circumstances, and that the peptone would therefore appear to be a complex of favouring and inhibitory factors; (3) that whole blood is better than serum and also better than washed blood, and that if the percentage of blood is below 2% the growth of some of the most delicate strains fails. Further, blood heated for a short time above 55°C. but not above 100°C. is superior to unheated blood. We wish to draw special attention to the importance of heating the blood, for this point has been overlooked by many of those who have recommended the addition of blood to media used for growing the gonococcus. The credit for introducing heated blood agar or "chocolate" agar has been wrongly given to Dr. Olga Povitzky (1912) by Coca and Kelley (1921), since this medium, as pointed out by Kristensen (1922) was described by Cohen and Fitzgerald (1910). The last-named particularly emphasized its value for growing bacilli of the type of Pfeiffer's bacillus. Our experience is that the superiority of heated blood agar over agar containing unheated blood is distinct for the growth of the gonococcus, as well as for that of Pfeiffer's bacillus, although in the case of the former the superiority is less marked. We have only come across one direct reference to this point in the literature of gonococcal culture, that of Ruediger (1919), who mentions it in a short paper on "Exclusion of Air in the Cultivation of the Gonococcus." Warren Crowe (1915), however, points out that the gonococcus grows well on his special "chocolate" agar—a medium in which the percentage of heated blood is much higher than in others described by this name.

Before discussing in detail the significance of these three elements in our medium it may be stated that as far as possible other conditions which undoubtedly play a part in the life of the micro-organism have been kept constant. It would seem probable from the work of Hitchens (1921) that the physical condition of the medium is of some importance. The publications of Wherry and Oliver (1916), Chapin (1918), Ruediger (1919), Kohman (1919), Rockwell (1921), and more particularly those of Novy (1925) and his collaborators, emphasize the insufficiently appreciated effect of gaseous environment on bacterial growth. Lastly the importance of reaction, which has been widely appreciated of recent years, is probably as much to be considered in cultures of the gonococcus as in those of any other bacterium.

The initial reaction of all cultures was rigidly controlled and the conditions of gaseous environment were roughly similar; but there is no doubt that

variations in the physical state must have existed in the different media used, and that this is an irregular factor in our experiments. In considering the three principal elements in our medium and attempting to understand the part which they play the blood is taken first.

Effect of Blood on Culture of the Gonococcus.

Of the three elements this is the one which has been least investigated. It seems possible that the favouring effect is actually due to the blood-pigment —the altered hæmoglobin of heated blood. If this assumption is correct then it is likely that the pigment acts in some way as a catalyst of processes of oxidation, somewhat in the same way as Fildes (1921) has suggested that it does in the case of the influenza bacillus. The superiority of whole blood may be due to the additional colloid derived from the serum. Certainly in many of the media recommended for the growth of the gonococcus the procedure is such as to ensure the presence in the medium of some blood-pigment derived from meat extract or otherwise. For instance, blood is added directly to Cole and Lloyd's (1916) medium and no finer filter than glass-wool is used subsequently, *i.e.* the medium will contain minute coagula of heated blood. Again, Huntoon's (1918) " hormone " agar is prepared by adding agar to the meat extract before the latter has been coagulated by heat, and fine filtration is avoided subsequently. We have not performed any crucial experiments, however, with regard to the importance of blood-pigment, and cannot therefore say more. It is interesting to note that many of the best results recorded with serum agar media have referred to placental serum (Martin, 1911; Stross, 1905). This may have some unusual component or quality.

Effect of Meat Extract.

The method of approaching this question was to compare the growths resulting from both heavy and light inoculations of four strains of gonococcus on a series of heated blood-agar plates prepared in the manner described above. The series included plates made with a basis of meat extract peptone agar, others with a basis of peptone agar without meat extract, and others again with a basis of peptone agar to which various substances had been added as substitutes for meat extract. The substitutes investigated were: (*a*) K_2HPO_4 and Na_2HPO_4, (*b*) Ringer's and Tyrode's salt solutions, (*c*) solutions of the ash obtained from inspissated meat extract which were added to the medium in such amounts as to give a salt-content similar to that of the original meat extract. The result of these investigations was to show—(1) that better growth was always obtained with blood peptone agar + phosphate, Ringer solution, Tyrode solution or ash suspension than in blood peptone agar alone; (2) that the media in which ash suspensions or Tyrode solution formed the basis gave better growths than the others, but were rarely equal to those obtained with the unaltered meat extract. These results were frequently repeated, and justify the conclusion that an important element in the value of meat extract for promoting growth of the gonococcus is its salt content. There must, however, be some other element which is presumably organic in character.

Attempt to Define the Important Organic Element in Meat Extract.

With this in view the effects of various precipitants and of dialysis were investigated. When meat extract was precipitated with 75% alcohol, the filtrate after concentration and restoration to the original bulk was equal to the untreated meat extract. The filtrate obtained after precipitation with 90% alcohol was less good, and the filtrate obtained after complete precipitation with colloidal iron was valueless. In experiments on dialysis the favouring substance appeared to be partly dialysed and partly retained. In fact, any clear-cut separation of the substance seemed particularly difficult, which is not surprising in view of Mueller's (1922) failure to isolate the substance in meat extract favourable to the streptococcus. He found this to be extracted by charcoal and capable of substitution by an acid hydrolysate of casein, but was unable to isolate it in spite of a great deal of elaborate work. All that we can infer from our own work is that the second favouring element in meat extract is some organic compound or compounds less complex than the albumins, globulins and higher protein molecules.

EFFECTS OF PEPTONE AND AMINO-ACIDS.

It is to this part of our subject that most time has been devoted, and it is in regard to it that we believe our conclusions to be most firmly founded.

There has been a tendency in the last ten years to adopt tryptic digests as a basis for bacteriological media. This was originally a war expedient (Douglas, 1916), but such digests have also been specially recommended as bases for the growth of delicate bacteria (Cole and Lloyd, 1916). It was shown, however, by the late Dr. G. A. Wyon and one of us (McLeod and Wyon, 1921; Wyon and McLeod, 1923) that tryptic digests in any considerable concentration inhibited the growth of delicate bacteria unless these were protected by the addition of various colloids. Torrey and Buckle (1922), investigating the culture of the gonococcus, have to some extent confirmed these observations, since they found that media rich in amino-acids were never superior to a medium containing 1½% of peptone, and that for some strains they were inferior. Apart from the inhibitory effect of tryptic digests, however, Wyon (1923) noticed that even peptone solutions contained substances inhibitory to the growth of meningococcus, which might be removed by treating the peptone solutions with charcoal.

In the course of an independent investigation with regard to the nature of the factor in ascitic fluid which promoted the growth of the gonococcus, we obtained evidence which strengthened Wyon's conclusion with regard to the existence of factors inhibitory for delicately growing bacteria in peptone solutions. We have not been able to find any record of investigations directly bearing on this point, but it is noteworthy that Vedder's (1915) starch medium, which he so strongly recommends for the growth of the gonococcus, contains no peptone. We have been able to grow gonococci on this medium, but the growths were not particularly good. There are, however, one or two papers in which some comment is made on the significance of the amount of peptone in media used for the gonococcus. Thus,

North (1909) claims that one of the reasons for the superiority of his agar-gelatine medium is that it contains 2% of peptone (Witte's). Thalmann (1900), however, states that amounts of peptone greater than 1% did not give any increased growth of the gonococcus. His observations referred to primary culture of the gonococcus on a simple meat extract agar. For the most part, however, investigators dealing with the cultivation of the gonococcus have taken for granted as a suitable basis a meat extract agar containing 1% of peptone and have studied the effect of various additions to that medium.

Our observations on the effect of the presence of various percentages of ascitic fluid on the growth of the gonococcus in solutions of peptone of different strengths gave unexpected results. One out of many such experiments is set out in Table I. Series of cultures were prepared in which various basal fluid media were diluted with different percentages of ascitic fluid. The figures in the columns show the amount of growth which had taken place in the fluid media after 40 hours' incubation as indicated by the profusion of growth in subcultures on a suitable solid medium.

TABLE I.—*Effect of Different Concentrations of Ascitic Fluid and of Peptone.*

First gonococcal strain:

% of ascitic fluid added to fluid media.	Basal fluid media.						
	Distilled water.	Meat extract.	Meat extract + 0·1% peptone.	Meat extract + 0·25% peptone.	Meat extract + 0·5% peptone.	Meat extract + 1% peptone.	Meat extract + 2·5% peptone.
40	scanty	+	+	scanty	+	scanty	scanty
20	0	scanty	+	+	+	scanty	0
10	0	13	+	+	+	0	0
5	0	24	20	scanty	scanty	0	0
2·5	0	1	scanty	0	0	0	0
1	0	0	scanty	0	0	0	0
0	0	0	0	0	0	0	0

Second gonococcal strain:

40	4	+	++	scanty	+	+	0
20	0	scanty	++	scanty	4	0	0
10	0	1	++	scanty	0	0	0
5	0	0	+	0	0	0	0
2·5	0	0	scanty	0	0	0	0
1	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0

The fluid media were incubated at 37° C. for 24 or 40 hours before subculture.
In this and the following tables the meanings of the signs are as follows:
++ = confluent growth.
+ = partly confluent growth.
scanty (sc.) = discrete colonies too numerous to be readily counted.
Numbers = number of colonies which developed.
C. = contamination.
They represent 24 hours' growths on "chocolate" agar inoculated from the fluid media.

The specimen of ascitic fluid used in this work showed in a rather marked degree the phenomenon to which Murray and Ayrton (1925) have drawn attention in a recent paper, that is, a marked tendency to become alkaline on standing. Not only was this the case, but an alkaline reaction was re-established after about 24 hours if the fluid was neutralized with acid. All media were adjusted to pH 7·4–7·6 at the outset, and since growth of both strains occurred in the undiluted ascitic fluid, and also, as shown in the table, in ascitic fluid diluted with 60 % of distilled water, this peculiarity of the ascitic fluid does not seem to invalidate in any way the general results. The experiment shows that meat extract alone is a good medium for growth of the gonococcus when enriched with small percentages of ascitic fluid, but that it is greatly improved by the addition of amounts of peptone much smaller than those used in ordinary laboratory media. When, however, the amount of peptone is raised, certain inhibitory elements at once come into evidence, since more and more ascitic fluid is required, till with 2½ % of peptone growth is completely inhibited, or no better than can be obtained with a similar percentage of ascitic fluid diluted with water.

The only explanation of these results which seemed to us to be possible was that the peptone consisted either of a mixture of favouring and inhibitory elements, or of elements favouring growth at low dilutions, but inhibiting it when more concentrated. One of the functions of the ascitic fluid was apparently to protect the bacteria from the inhibitory elements in the peptone. If this assumption of a protective function for the ascitic fluid were a correct one, we thought that it should be possible to show similar effects with other colloid-containing fluids such as serum and blood. With these fluids the results were never so definite as with ascitic fluid, but they were similar in character. Some of the irregularities in experiments with serum were doubtless due to the varying inhibitory effect of animal serum on the growth of the gonococcus. In a number of experiments with different animal sera inhibitory effects were observed when the media contained more than 10 % of serum and could be abolished by heating the serum. Similar observations have been recorded by Stross (1905). Heated blood, on the other hand, had so marked a favouring effect that the influence of varying concentrations of peptone appeared to be sometimes masked. Some of the more characteristic results with additions of these substances to media of varying peptone concentration are shown in Tables II and III:

TABLE II.—*Effect of Increasing Amounts of Peptone on the Growth of Gonococci in Fluid Media containing Serum.*

% of serum.	5% peptone.	2% peptone.	1% peptone.	0·5% peptone.	0·2% peptone.	0·1% peptone.
10	0	+	+	+	+	+
5	0	0	+	+	+	+
2	0	0	0	+	+	+
1	0	0	0	0	+	+
0	0	0	0	0	0	0

TABLE III.—*Effect of Increasing Amounts of Peptone on the Growth of Gonococci in Fluid Media containing Heated Blood.*

% of blood.	5% peptone.	2% peptone.	1% peptone.	0·5% peptone.	0·2% peptone.	0·1% peptone.
10	+	+	+	+	+	+
5	+	+	+	+	+	+
2	scanty	moderate	+	+	+	+
1	0	scanty	scanty	scanty	scanty	+

TABLE IV.—*Effect of Increasing Concentrations of Different Peptones on the Growth of the Gonococcus in Fluid Media containing Serum.*

A. *Parke-Davis' Peptone.*

% of serum.	5% peptone.	2% peptone.	1% peptone.	0·5% peptone.	0·2% peptone.	0·1% peptone.
10	0	0	++	++	++	++
5	0	0	+	++	++	++
2	0	+	0	++	++	++
1	0	0	0	0	scanty	scanty

B. *Chapoteaut's Peptone.*

10	0	0	0	+	++	++
5	0	0	0	++	++	0
2	0	0	15	++	++	++
1	0	0	0	0	0	scanty

C. *Witte's Peptone.*

10	0	+	+	++	++	++
5	0	+	+	+	+	+
2	0	0	0	scanty	+	scanty
1	0	0	0	scanty	scanty	scanty
0	0	0	0	0	0	0

TABLE V.—*Effect of Increasing Concentrations of Peptone on the Growth of Pneumococcus in Fluid Media containing Serum.*

% of serum.	5% peptone.	2% peptone.	1% peptone.	0·5% peptone.	0·2% peptone.	0·1% peptone.
10	+	+	+	+	+	+
5	0	+	+	+	+	+
2	0	+	+	+	+	+
1	0	scanty	+	+	+	+
0	0	0	0	0	0	0

Up to this point all investigations had been made with Parke-Davis peptone which we use for the preparation of routine media. For purposes of comparison two others were tested in order to determine whether the effects observed were common to different peptones. The results with Chapoteaut's and Witte's peptones, using serum as a protective, are shown in Table IV. All

TABLE VI.—*Influence of Taurine and Tryptophane in the Presence of Varying Percentages of Blood.*

	Strain 4.						Strain 8.					
	0·1% blood.	0·25% blood.	0·5% blood.	1% blood.	2% blood.	5% blood.	0·1% blood.	0·25% blood.	0·5% blood.	1% blood.	2% blood.	5% blood.
2 % taurine	0	50	+	+	++	++	0	sc.	++	++	++	++
1 % „	0	30	+	++	++	++	0	++	++	++	++	++
0·5 % „	0	60	++	++	++	++	0	sc.	++	++	++	++
0·25 % „	0	sc.	++	++	++	++	0	+	++	++	++	++
0·1 % „	0	30	++	++	++	++	0	sc.	++	++	++	++
1 % tryptophane	0	0	0	0	0	0	0	0	0	0	0	0
0·5 % „	0	0	30	100	sc.	sc.	0	0	10	sc.	+	++
0·25 % „	0	sc.	+	+	++	++	3	100	+	++	++	++
0·1 % „	0	19	++	++	++	++	0	100	++	++	++	++
Control broth	0	0	sc.	+	++	++	0	sc.	++	++	++	++
„ „	0	0	+	+	++	++	0	7	+	++	++	++
„ „	0	0	++	++	++	++	0	4	+	++	++	++

TABLE VII.

		Total number of tests.	Tests valid for showing favouring effects.*	Number and percentage of tests in which amino-acid favoured growth.	Tests valid for showing inhibitory effects.†	Number and percentage of tests in which amino-acid inhibited growth completely.	Average inhibitory concentration of amino-acid in experiments showing inhibition.
Group I.	Glycine	47	19	0	28	26, or 93%	0·16%
	Tryptophane	83	32	9, or 28%	61	50, or 82%	0·24%
	Phenylalanine	16	5	2, or 40%	13	12, or 92%	0·26%
	Cystine	14	3	1	12	7, or 58%	0·09%
	Asparagine	5	1	1	5	4, or 80%	0·22%
	Alanine rac.	9	2	1	8	7, or 87%	0·35%
Group II.	Histidine	13	3	0	10	3, or 30%	0·27%
	Arginine	4	0	0	4	2, or 50%	0·4 %
	Lysine	5	1	0	4	1, or 25%	0·25%
	Glutamic acid	4	0	0	4	1, or 25%	0·1 %
Group III.	Alanine d.	63	26	7, or 27%	43	15, or 35%	0·31%
	Aspartic acid	30	11	7, or 63%	27	14, or 52%	0·34%
	Taurine	56	20	11, or 55%	43	7, or 16%	0·48%

* Those in which growth failed in the controls. † Those in which growth occurred in the controls or with smaller amounts of the same amino-acid.

three peptones tended to inhibit growth in the higher concentrations, but the protective effect of serum was least obvious in the case of Chapoteaut's peptone. Lastly, an attempt was made to find out if this peptone sensitiveness was peculiar to the gonococcus. The results obtained with a pneumococcal strain are shown in Table V. They are similar to but much less marked than those obtained with the gonococcus.

Having obtained consistent evidence, therefore, that the ordinary bacteriological peptones might exert an inhibitory as well as a favouring effect on gonococcal growth, and having shown previously that tryptic digests of serum and casein might be distinctly inhibitory to the gonococcus, the next step indicated was to determine the effects of individual amino-acids on the growth of this bacterium.

The amino-acids used were obtained from Messrs. Hoffmann la Roche, and we are indebted to Dr. J. Gordon (Gordon and McLeod, 1926), who was also working with them, and to Mr. E. R. Dawson for the control of their purity. Since none of the gonococcus strains at our disposal were capable of growing in ordinary peptone broth, we sought to devise a medium which would grow the more hardy strains and fail to support the more delicate ones. Such a medium we considered would be suitable for throwing into relief the favouring or inhibitory effects of the added amino-acids, since a favouring substance might be expected to promote growth of a delicate strain which failed in the control, and an inhibitory one would prevent the growth of more hardy strains which developed readily in the control tubes. Such a medium we found in meat extract + 1% of peptone and 0·5% of NaCl reinforced with 0·1%-0·3% of defibrinated rabbit's blood and heated for 15 minutes at 70°C.

Of the eleven strains of gonococcus used, 1, 2, 5, 6 and 9 grew fairly constantly in the control tubes, strains 4, 7 and 11 invariably failed to grow, and strains 3, 8 and 10 grew irregularly. These strains were obtained from the urethral discharge in cases of acute gonorrhœa. None of them grew on the ordinary laboratory agar. All had a typical morphology and fermented only glucose.

Thirteen amino-acids were investigated—glycine, d-alanine, racemic alanine, taurine, l-cystine, aspartic acid, glutamic acid, asparagine, d-lysine, arginine, phenylalanine, l-tryptophane and l-histidine. As it was not possible to examine more than two or three amino-acids and a few strains of gonococcus in each experiment, and as the percentage of blood used was varied from experiment to experiment, and also the range of concentrations of the amino-acids, our results are not suitable for an exact analysis. The nature of the influence of the amino-acids will be best appreciated from the general summary of our findings presented in Table VII, and from the reproduction of a couple of representative experiments—Tables VI and VIII.

The effects of the amino-acids are governed to a considerable extent by the amount of blood present in the medium. This is illustrated in Table VI, in which the effects of tryptophane and taurine on the growth of two strains of gonococcus in media containing varying percentages of blood are shown. Here, just as in the case of peptone solutions of various concentration, it is seen that as far as tryptophane is concerned the less there is of amino-acid the less blood is required to ensure growth. It is seen, however, also, that with

(Taurine	56	20	11, or 55%	43	7, or 16%	0·48%

* Those in which growth failed in the controls. † Those in which growth occurred in the controls or with smaller amounts of the same amino-acid.

a certain amount of blood present the smaller percentage of amino-acid will favour growth, but that however much blood is added protection will not be afforded from high concentrations of this amino-acid—above 0·5%. No inhibitory effects are observed with taurine in this experiment. If, however, gonococcal strains are chosen which are hardier than those used in the experiment recorded in Table VII, *i.e.* such as will grow in control tubes containing 0·1%–0·2% of blood, it is found that inhibitory effects are obtained with taurine also and, indeed, with all amino-acids tested in media containing less than 0·2% of blood.

TABLE VIII.—*Effect of Taurine, Aspartic Acid, Histidine and Glycine on Growth of Gonococcus.*

		Strain 1.	Strain 2.	Strain 3.	Strain 4.	Strain 6.	Strain 8.
Blood broth + taurine	1%	++	++	++	+	++	++
" "	0·75%	++	++	++	1	++	++
" "	0·5%	++	++	++	25	++	+
" "	0·25%	++	++	70	0	++	++
" "	0·1%	++	++	sc.	0	++	++
Blood broth + aspartic acid	0·5%	50	++	++	0	++	sc.
" "	0·25%	150	++	++	0	++	+
" "	0·1%	sc.	0	++	0	++	++
" "	0·05%	++	++	0	0	+	++
Blood broth + histidine	0·5%	++	1	0	0	++	++
" "	0·25%	++	++	0	0	++	++
" "	0·1%	++	++	0	0	++	++
" "	0·05%	++	++	0	0	++	2
Blood broth + glycine	0·5%	0	0	0	0	0	0
" "	0·25%	0	0	0	C.	+	0
" "	0·1%	50	++	0	0	++	0
" "	0·05%	55	++	0	0	++	++
Controls. Blood broth only		0	++	+	0	++	++
" " "		++	++	0	0	++	++
" " "		++	++	0	0	++	++

The percentage of blood added to the fluid media before heating was 0·23% in this experiment.

In view of these facts the amino-acids are classified in three groups in Table VII, in which the general summary of the results of our tests is presented.

Group 1 consists of those amino-acids which in higher concentrations produce inhibitory effects on the growth of gonococcus with great constancy, but which in low concentrations may favour the growth of such gonococci as grow only in presence of a considerable amount of blood.

Group 2 consists of those amino-acids which do not produce inhibitory results with any great constancy, and which have not been shown to favour growth.

Group 3 consists of amino-acids which, although they may inhibit in high concentrations when there is little blood present, usually favour growth even in high concentration when 0·5% or more of blood is present.

Those amino-acids which were not tested sufficiently often to obtain thoroughly reliable figures are printed in italics in the table. It will be noted

that a very low inhibitory figure is obtained with cystine, but that only 58% of inhibitory results are recorded. This is probably due to the difficulty encountered in keeping anything more than 0·1% of cystine in solution, and on this account these results are less to be relied on than the others.

The actual difference between Groups 1 and 3 in respect of the figures shown in the last column for the percentage of the amino-acids causing complete inhibition is not large. But it must be remembered that the averages for Group 1 are very nearly actual averages for all tests done, whereas for Group 3 in 50% of the tests or more the inhibitory concentration was not reached, and the true averages would actually be much higher.

A clearer view of the relative effects of amino-acids belonging to the different groups is obtained from the record of the experiment given in Table VIII. Thus it is seen that in the case of gonococcal strains failing to grow or growing poorly in the controls, better growth is obtained the larger the amount of taurine present. With glycine the strains tending to fail in the control tubes do not grow at all, and even those strains which grow well in the controls are inhibited when anything more than 0·25 % of amino-acid is present. With histidine, the amino-acid of Group 2, which appears in this experiment, there is neither a pronounced favouring nor an inhibitory effect.

Two other investigations have been made: (*a*) on the effect of the presence of two or more amino-acids simultaneously and (*b*) on the effect of the presence of colloids. When mixtures of alanine and taurine, alanine and tryptophane, taurine and tryptophane or of all three are added in various strengths to blood-broth media, the inhibitory effect produced is the sum of the inhibitory effects of the various amino-acids present in the mixture, *e. g.* a mixture containing 0·2% each of tryptophane and alanine, where these acting separately have been found inhibitory, in concentrations 0·3% and 0·4% respectively will be found unsuitable for growth; whereas partial inhibition only will be obtained by a mixture of 0·4% each of tryptophane and taurine under experimental conditions in which taurine is showing no inhibitory effect in a concentration of 0·8%, but tryptophane is producing partial inhibition at concentrations of 0·4%.

Hitchens (1921) had described cultures of gonococcus in fluid media containing small percentages of agar with a basis of extract of putrid meat. We repeated his work, using laboratory peptone broth as a basis in order to ascertain the effect of the variation in the colloid content of the medium. We had no success in growing any strain of gonococcus unless blood was added, except in a few experiments in which taurine was present. We did find, however, that in the presence of small percentages of agar considerably less blood was required to ensure growth. This appeared to be due to a better distribution of the heated blood through the medium in the presence of the agar. Without agar it tended to sediment to the bottom of the tube. Also the degree of protection from amino-acids afforded by a mixture of blood and agar was greater than that afforded by the same amount of blood alone.

DISCUSSION.

The chief practical result of this work seems to us to consist in showing, first, that great discrimination must be exercised in using tryptic digests as a

basis for media intended for the growth of the gonococcus; and second, that by adding amino-acids of the third group described to media, or better, by substituting such for part of the peptone, specially good media for the growth of the gonococcus are likely to be obtained. Amongst these, taurine, amino-ethyl-sulphonic acid ($NH_2.CH_2.CH_2.SO_2OH$), which is not an amino-acid in the exact sense of the term, is likely to give the best results. This is the more interesting since, according to the work of Koser and Rettger (1919), taurine is inferior to most amino-acids when used as the sole source of nitrogen in a medium.

Looked at from the theoretical standpoint these results appear to go some way towards the explanation both of the irregular results obtained in the cultivation of the gonococcus in empirical media, usually successful, and also of the constant failure of the gonococcus on ordinary nutrient agar.

Although it is unlikely that in media in which blood is incorporated any one amino-acid will be present in sufficient concentration to exert an inhibitory effect, yet it is not unlikely that in some such media the added effects of a number of the definitely inhibitory amino-acids of the first group may prevent growth.

There is a very close resemblance between other delicately growing bacteria in their sensitiveness to various amino-acids, as shown in the observations of Gordon and McLeod (1926), and the gonococcus. With the gonococcus, however, the difference between inhibitory and favouring amino-acids is less sharp. This may be due to the necessity of adding blood, etc., in order to ensure the growth of the gonococcus.

The explanation of the failure of the gonococcus to grow on nutrient agar would appear to depend on the fact that this micro-organism requires heated blood or some colloid with similar properties to enable it to utilize amino-nitrogen, and that, further, there must be a definite ratio between amino-nitrogen and favouring colloid. The ordinary peptone meat extract media are unduly rich in amino-nitrogen relatively to their contained blood-pigment, derived from the meat extract, and do not therefore permit growth.

GENERAL CONCLUSIONS.

1. The elements essential to the growth of the gonococcus appear to be the following:

   (1) Some source of amino-nitrogen and of carbon.

   (2) Salts such as are present in meat extract.

   (3) A colloid which is capable of protecting the organism from undue concentrations of certain amino-acids and of enabling it to assimilate these and others. Blood heated between 60° and 100° has proved itself far superior to other colloids tried in this respect.

2. Unless the gonococcus is protected by suitable colloid it is inhibited even by peptone solution; for it is observed that growth occurs in the presence of much lower concentrations of colloid if the peptone is used in a concentration of $\frac{1}{2}\%$ to $\frac{1}{10}\%$ rather than 1% or 2% as usually recommended for bacteriological media.

3. On investigating the effect of amino-acids on the growth of the gonococcus it is found that they are roughly classified into three groups:

(*a*) Amino-acids inhibitory in concentrations above 0·5% irrespective of the amount of protecting colloid, but sometimes showing favouring effects in low concentrations, 0·05% to 0·2%, glycine, tryptophane, phenylalanine, and perhaps cystine; (*b*) amino-acids inhibitory only where small amounts of protective colloid are present but otherwise indifferent; (*c*) amino-acids which may be inhibitory in presence of low concentrations of colloid, but which usually favour growth when more than 0·25% of heated blood is present in the medium, d-alanine, aspartic acid and taurine. The last, which is not an amino-acid in the true sense, shows the most powerful favouring effect.

4. That a mixture of amino-acids in which no single one is present in an inhibitory amount produces inhibition of growth owing to summation of inhibitory effects, and that in any case the gonococcus appears to be unable to utilize amino-nitrogen unless a certain ratio of blood, or equivalent colloid, to amino-nitrogen is maintained. The failure of the gonococcus to grow on ordinary nutrient agar would, in view of this conclusion, depend on an unduly low ratio of suitable colloid to amino-nitrogen.

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EXPERIMENTS ON STREPTOCOCCAL HÆMOLYSIN AND THE ANTAGONISTIC ACTION OF BILE AND BILE-SALTS.

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In the course of an investigation of the nature and properties of the hæmolytic substance which is produced under appropriate conditions by the *Streptococcus hæmolyticus*, certain observations have been made which are of interest in view of the current views on the nature of this substance.

At various times evidence has been adduced that it is related to the higher fatty acids (Connell and Holly, 1920), to glucosides such as saponin (Sherwood and Downs, 1925), and according to Ruediger (1907) the hæmolysin is associated with the globulin fraction of the medium in which it is produced.

Meader and Robinson (1920) maintain that one of the substances from which hæmolysin is elaborated contains phosphorus, and that the other is an unknown body to which certain properties are attributed. Orcutt and Howe (1922) have shown that on the addition of fat in the form of cream to staphylococcal cultures hæmolytic substances are formed, and suggest that they are due to the action of lipase. This finding suggested the possibility of a connection between the lipase action of streptococcus and the hæmolytic power of cultures of this organism.

COMPARISON OF LIPOLYTIC ACTIVITY OF STREPTOCOCCAL AND STAPHYLOCOCCAL CULTURES WITH HÆMOLYSIN TITRE OF THE CULTURES.

The presence of a lipolytic enzyme in cultures of hæmolytic streptococci has been demonstrated previously (Stevens and West, 1923) and in cultures of staphylococci by Michaelis and Nakahara (1923). In the following experiment it is shown that an emulsion of staphylococcus can hydrolyse ethyl butyrate, and moreover the enzyme appears to be mainly of endocellular origin. That this is the case for pneumococcus has been suggested by Avery and Cullen (1920), who find an increased lipase action in solutions of pneumococcus in bile.

It is not possible to use this method in the preparation of lipase from staphylococcus and streptococcus, nor is it desirable, since bile has a marked activating effect on lipase.

For estimating the endolipase, suspensions of staphylococci were disintegrated by repeatedly freezing and thawing in liquid air.

Staphylococcus aureus was grown on 1% peptone bouillon agar slopes for 18 hours. The growth was washed off in a buffer (phosphate) solution pH 7·6 (Clark, 1922). The methods used in the estimation of lipase were those described for pancreatic lipase by Platt and Dawson (1925). Ethyl butyrate was used as substrate, and the lipase action was determined after 24 hours'

incubation at 37° C. by titration to pH 7·6 with N/50 NaOH, using phenol red as indicator. The reaction was stopped by cooling in ice-cold water. All the experiments were carried out in duplicate, the results being expressed as the mean of these estimations, corrected for controls for hydrolysis by buffer solutions alone. Control experiments were also carried out with test samples containing no substrate.

TABLE I.

Mixtures.	Hydrolysis. c.c. N/50 NaOH.
0·1 c.c. ethyl butyrate, 0·5 c.c. toluol plus (1) 2 c.c. phosphate emulsion of staphylococcus	0·93 c.c.
(2) 2 c.c. phosphate emulsion was centrifuged and supernatant fluid tested	0·70 c.c.
(3) Centrifuged deposit from (2) diluted with 2 c.c. phosphate and tested	0·54 c.c.
(4) No. (3) repeatedly frozen and thawed	1·37 c.c.

In the absence of toluol an increased amount of lipase was apparent in mixtures similar to (1) above. This on investigation was found to be due to growth of the organism in the phosphate mixture. The results set out in the foregoing protocol show that the lipase action obtained is increased if the washed residuum is disintegrated, the amount of lipase present in the washed residue (3) being probably liberated by autolysis of the organisms.

The lipase activity of hæmolytic and non-hæmolytic strains of streptococci were then compared with that of staphylococcus. The organisms were all obtained from hospital cases and were grown in 6 c.c. of 20% serum bouillon, the serum (ox) having been heated for 30 minutes at 60–65°C. in order to destroy serum lipase. Parallel series of cultures heavily inoculated with these organisms were incubated for 24 hours, one series containing 0·5% ethyl butyrate. In the latter series the amount of hydrolysis was estimated as described above, whilst the hæmolysin titre was determined in cultures not containing ester. The amount of growth as estimated by subculture to heated blood-agar plates showed that the ester had no marked inhibitory action to growth under these conditions. The hæmolysin titre was determined by adding varying amounts of culture to 1 c.c. of a 3% suspension in saline of ox red blood-corpuscles and incubating for 2 hours.

TABLE II.

Organism.	Hæmolysin titre.			Hydrolysis. N/20 NaOH.	Growth.	
	0·2 c.c.	0·1 c.c.	0·05 c.c.		No ester.	Ester.
Staphylococcus aureus	−	−	−	0·52 c.c.	++	++
"Goode" (strep.)	+++	++	+	0·47 c.c.	++	++
C.B. (strep.)	−	−	−	0·32 c.c.	++	++
F. (strep.)	−	−	−	0·06 c.c.	++	++

++++ = complete hæmolysis; +++ = almost complete hæmolysis; ++ = partial hæmolysis; + = trace of hæmolysis; − = no hæmolysis.

Table II shows that, whilst *Staphylococcus aureus* has a higher lipase content than streptococcus "Goode," the former organism has no hæmolysin. Moreover, the hæmolytic streptococcus "Goode" contains only slightly more lipase than the non-hæmolytic strain "C.B."

These results, therefore, indicate that there is no connection between the

lipolytic activity and the hæmolytic titre of the cultures employed. It is therefore suggested that the streptolysin is not produced by the action of the bacterial lipase on fatty substances present in the culture media. Furthermore, Meader and Robinson (1920) find that the removal of the ether-soluble fraction of bouillon does not reduce the value of the medium for streptolysin production. This observation has been repeatedly confirmed in the course of the present investigation.

EFFECT OF BILE AND BILE-SALTS ON THE HÆMOLYSIN.

If the formation of streptolysin were in any way dependent on the lipase activity, it should be possible to effect an acceleration of the hæmolysin production by the addition of bile-salts.

Bile-salts, however, markedly inhibit the growth of the streptococcus, and any observations of the possible effect of bile-salts on streptococcal hæmolysin and on lipase are complicated by this fact when cultures are investigated.

The present investigation is therefore confined to the examination of the influence of bile-salts on the hæmolysin already present in the culture under conditions in which further growth is excluded.

It was found that the addition of bile and bile-salts to cultures containing hæmolysin removed the hæmolysin. This fact does not necessarily imply that lipase does not enter into the production of the hæmotoxin, but rather that some other reaction between formed hæmolysin and the added bile and bile-salts has taken place.

A hæmolytic streptococcus ("C.S.M.") isolated from a fatal case of traumatic meningitis was grown in (1) a 1% peptone-bouillon medium and (2) a 1% peptone-bouillon medium to which 20% ox serum had been added. Parke-Davis peptone was used in the preparation of the media. Hæmolysin was produced in both media, but was much more marked in the serum peptone-bouillon than in the medium containing no serum.

It was found that in an 18-hours-old peptone-bouillon culture, containing only a small amount of hæmolysin, all hæmolytic activities were removed by 5 to 10 minutes' incubation at 37° C. after the addition of 0·1% sodium desoxycholate, 0·2% sodium taurocholate or 1% of bile. Freshly prepared 10% solutions of sodium desoxycholate and sodium taurocholate were made in distilled water, and the requisite amounts were added to the cultures. The samples of bile used were ox bile and human bile obtained *post mortem*. One of the samples of human bile was obtained during life.

TABLE III.—*Presence of Hæmolysin in 18 Hours' Peptone-Bouillon Cultures of Streptococcus after Addition of Bile and Bile-Salts.*

Culture to which has been added—	Hæmolysin titre amounts of culture.		
	0·2 c.c.	0·1 c.c.	0·05 c.c.
0·05% sodium desoxycholate	++	+	−
0·1% " "	−	−	−
0·1% sodium taurocholate	++	+	−
0·2% " "	−	−	−
0·5% bile	++	+	−
1% "	−	−	−
Culture alone	+++	++	+

1 c.c. of 3% ox red blood-corpuscles in each tube, incubated and read at end of 2 hours.

Amounts of bile and bile-salts less than the amounts given above did not generally inactivate the hæmolysin, but no hard and fast rule can be given, as the amount required appeared to be directly proportional to the amount of hæmolysin in the culture.

Larger amounts of bile and bile-salts were needed to neutralize the hæmolysin in serum peptone-bouillon culture—viz. 0·5 % sodium desoxycholate, 1 % of sodium taurocholate and 5 % of bile.

The hæmolytic titre of this culture was—

0·2 c.c.	0·1 c.c.	0·05 c.c.	0·01 c.c.	0·005 c.c.
++++	+++	++	+	—

These results are all the more interesting since bile and bile-salts are themselves hæmolytic when used in concentrations greater than those described in these experiments, or when used in similar concentrations in simpler solvents such as 0·85 % salt solution. Controls of medium and bile-salts, etc., in the concentrations stated were always put up, but did not show hæmolysis.

Bile and bile-salts added to a filtered culture containing hæmolysin showed the same inhibitory effect.

Connell and Holly (1920) have put forward the suggestion that streptolysin is an unsaturated fatty acid. The behaviour of oleic acid and sodium oleate towards bile-salts does not support this idea, inasmuch as it is found that the addition of bile-salts does not inhibit the hæmolytic activity of sodium oleate, and so far as can be ascertained the hæmolytic power of mixtures of sodium oleate and sodium desoxycholate is the sum of their separate hæmolytic activities. A similar conclusion has been reached in studying the effects of adding bile-salt to saponin.

DISCUSSION.

The evidence presented above suggests that the production of streptolysin does not run parallel to the amount of lipase in the bacteria. Further, the effect of bile-salts on oleic acid and saponin is so different from their effect on streptolysin that it is reasonable to assume that the streptolysin is neither of the same nature as saponin nor an unsaturated fatty acid. This effect of bile on streptolysin should be considered in assessing the value of one of the current theories with regard to the ætiology of pernicious anæmia. In the work of Hurst (1925), and Venables and Knott (1924), it is suggested that pernicious anæmia is caused by hæmolysin production by streptococci which are to be found in the small intestine. The main evidence adduced in support of this viewpoint is firstly that hæmolysis does occur in the body in that disease, and secondly that hæmolytic streptococci may be isolated from duodenal fluids obtained from cases of pernicious anæmia. An achlorhydria is a constant feature in pernicious anæmia, and it is assumed that on this account streptococci are able to pass from the mouth to the duodenum. There is no doubt that this is so. McLeod and Wheatley (1926), however, have shown that actively hæmolytic streptococci are rare in the duodenum in pernicious anæmia, and their work, besides that of many other observers,

indicates that other bacterial hæmolysins should be considered in this connection.

It seems possible, in view of the above results, that there is a sufficient concentration of bile in the duodenum to neutralize any streptococcal hæmolysin which is likely to be produced there. At times there may be no bile present in the lumen of the duodenum, but even then it is probable that some will be present in the folds of the mucosa.

The objection may be raised that the infection is localized in the tissues, and that the organisms penetrate sufficiently to be protected from the action of bile. It is probable, however, that the bile will diffuse as deeply into the duodenal wall as the streptococci have ever been shown to penetrate. It might be supposed that a bile-hæmolysin compound entered the blood-stream and was dissociated there with the liberation of an active hæmolysin. No attempt has been made as yet to separate the hæmolysin from the bile, if such a separation be possible.

SUMMARY AND CONCLUSIONS.

1. Streptolysin production does not appear to be determined by the lipase content of the bacteria concerned.

2. Streptolysin action is inhibited by bile and bile-salts. This phenomenon is not observed with oleic acid and saponin hæmolysis.

3. It is difficult to reconcile the neutralization of streptococcal hæmolysin by bile with a theory of pernicious anæmia based on the hæmolytic activity of streptococci located in the duodenum.

I desire to express my thanks to Dr. Platt and Mr. Dawson for their valuable assistance with the lipase estimations, to Prof. J. W. McLeod for his constant help and interest, and to the Medical Research Council for a grant in aid.

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EXCITATION OF BACTERICIDAL FLUORESCENCE BY MEANS OF X-RAYS.

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IN 1917 H. S. Newcomer (1917) reported experiments which proved that the crystal fluorite has the property of emitting bactericidal rays under X-ray excitation. He pointed out that this might be analogous to the well-known examples of visible fluorescence exhibited by many bodies under the influence of ultra-violet or X-rays. While emphasizing the potential therapeutic value of such fluorescent energy for bactericidal purposes, Newcomer was unable to find a more practical substance than fluorite, and, as far as published work goes, the matter does not seem to have advanced any further.

Repeated attempts to find a photo-chemical method of testing substances for bactericidal fluorescence under X-ray excitation have met with little success in our hands, and in the following experiments we have relied upon direct tests on living bacteria.

The aim of the research has been the discovery of a substance that fulfils the following conditions:

(1) It must be soluble in water. (2) It must show definite bactericidal fluorescence under X-ray excitation, as tested on living organisms *in vitro*. (3) It must be effective in dilutions which can safely be injected into the animal body. (4) It must be of low toxicity.

While we have not yet discovered the ideal substance, we have found that calcium phospho-tungstate fulfils the first two conditions and partly satisfies the third. The possibility of bactericidal fluorescence from the insoluble calcium tungstate was pointed out to us by Dr. L. H. Clark, Research Physicist of the Hospital. The insolubility of this salt being a practical disadvantage, we tested the soluble phospho-tungstate with sufficiently encouraging results to merit further research. Levy and West (1921) have shown that photographic plates specially treated with calcium tungstate are many times as sensitive to X-rays as untreated plates, the enhanced effect being due to fluorescence of ultra-violet rays. It might well be that such fluorescent rays would also have bactericidal properties, and might in fact be ultra-violet rays shorter than 2960 Å.U.

METHODS.

Our preliminary experiments were based on the work of Newcomer (referred to above), but, owing to differences in technique, our results are not strictly comparable with his. Newcomer used saline suspensions of *B. typhosus*, which he placed in holes of $\frac{1}{10}$ in. bore and 1 cm. depth drilled in a

crystal of fluorite. The crystal thus filled was exposed to X-rays from a Coolidge tube, actuated by a Snook interrupterless transformer with an alternative spark-gap of 9 in. and a 1 mm. aluminium filter covered with black paper.

In our experiments we used a crystal of fluorite hollowed out to a depth of 1 cm. in the form of a rounded cell of 3 cm. diameter, which was then polished. Similar glass cells were used as controls. The cells were made in the form described to avoid screening effects.

When considering the effect of fluorescent radiation on organisms, it is obviously desirable to obtain as even a distribution of energy as possible acting throughout the suspension. Since flourescence implies absorption of the exciting radiation, it was thought that a shallow cell without any angles would be an improvement on a cylindrical cell, in which there is bound to be a difference between the intensity of fluorescence at the top and bottom of the cell.

We have expressed our X-ray dosage in terms of the rad—the lethal dose for Jensen's rat sarcoma (Russ, 1918)—a dose more easily standardized than that employed by Newcomer.

B. coli communis was used for most of our experiments, as it has the advantages of being safe to handle and of behaving in a very constant fashion under direct ultra-violet irradiation. It can, moreover, be suspended in sterile water or saline without prejudice to its subsequent growth—an important point emphasized by Newcomer, who found that traces of broth would shield organisms from ultra-violet rays.

The following technique was adopted in the experiments described below:

(1) *Bacterial suspension.*—The most satisfactory suspension was made by adding 1 drop from a gauged pipette (0·025 c.c.) of a 24-hour broth culture of *B. coli* to 10 c.c. of sterile physiological saline. Such a suspension contains about 500,000 organisms per c.c. Agar cultures were found to be unsuitable for making the suspension, as it is impossible to guarantee the complete and homogeneous emulsification of the organisms.

(2) *Filling of the cells.*—The cells, sterilized by exposure to a mercury lamp, were filled by adding 1 c.c. of sterile saline and 1 drop (0·025 c.c.) of bacterial suspension. They were then covered with thin mica and exposed to X-rays.

(3) *Colony counts.*—Consistent bacterial counts, with an error of less than 5% ±, were obtained by withdrawing a volume of 0·2 c.c. from each cell with a sterile graduated 1 c.c. pipette controlled by a rubber teat. This was transferred at once to about 10 c.c. of melted agar at 45° C., thoroughly mixed by rolling the tube between the hands and poured into a Petri dish. Surface colonies were avoided by allowing the medium to set in the dish and then flooding the surface with a thin film of sterile melted agar. All the colonies therefore developed under slightly anaerobic conditions and were easy to enumerate. Duplicate plates were employed. With the above technique about 2000 colonies per plate developed in 24 hours in the untreated controls.

RESULTS.

(a) *Fluorite Cell.*

When using suspensions containing from 7000 to 15,000 organisms per c.c. we obtained consistently small reductions, but these were not big enough to eliminate the possibility of experimental error.

With suspensions containing 100 or less organisms per c.c. we obtained from 85–90% reductions, and although with weak emulsions experimental error tends to increase, this marked reduction was taken to be due to a positive bactericidal effect. As far as they are comparable, our results are in agreement with those of Newcomer, for though he seems to have obtained more striking reductions, we do not know the exact strength of his bacterial suspensions, and much depends on the initial number of organisms.

Table I shows a comparison between some of our results and one of Newcomer's, the comparison being in our opinion justified by the similarity of the control X-ray effect in each case.

TABLE I.

Observer.	Organism.	Strength of suspension.	Control cell reduction.	Fluorite cell reduction.	X-ray dose in rads.
Newcomer	*B. typhosus*	?	50%	95–98%	?
Authors	*B. coli*	5780 per c.c.	57%	67·5%	¼
,,	,,	89 ,,	61%	94·5%	¼

Allowance must be made for possible differences between various samples of fluorite as pointed out by Newcomer. The fact that we were using *B. coli* does not invalidate the comparison, since the members of the coli-typhoid group appear to behave very similarly under direct ultra-violet irradiation.

(b) *Glass Cell using Various Solutions.*

It was thought that solutions showing a marked absorption spectrum in bactericidal range of the ultra-violet (2960–2100 A.U.) might, under the influence of X-rays, be excited to emit bactericidal wave-lengths. Flavine, brilliant green, methylene-blue and other organic dyes showing absorption spectra in the ultra-violet were tested extensively in non-bactericidal dilutions, but failed to give an enhanced effect under X-rays. As far as these organic solutions are concerned, the absorption spectrum does not seem to justify any forecast as to probable ultra-violet fluorescence under X-ray excitation.

(c) *Glass-cell utilizing Calcium Phospho-Tungstate.*

A 5% solution of calcium phospho-tungstate was kindly prepared for us by Mr. W. Lawson, M.Sc.

The glass cell was filled with phospho-tungstate solution and bacterial suspension in the manner described and the X-ray exposure made. Controls were performed by substituting saline for phospho-tungstate and exposing to X-rays, and by filling a cell with phospho-tungstate and bacterial suspension omitting the X-ray exposure.

Table II sets out our results, on which we have based the following comments and interpretations:

(1) It will be noted that the direct bactericidal action of the X-rays as measured by the reduction in the saline control is, with one slight exception, about 62%.

(2) Newcomer has pointed out that the number of organisms surviving the action of a destructive agent is influenced by the laws of probability. 10% differences in the numbers of survivors in the region of 50% killed have little significance, whereas the same percentage reduction indicates progressively greater lethal efficiency as complete sterilization is approached.

(3) It will be noted that the effect was diminished not only by lowering the dose of X-rays, but also by reducing the strength of the phospho-tungstate solution. This result is in keeping with the theory that the bactericidal action is due to a fluorescent phenomenon. Intensity of fluorescence of a solution under steady illumination by the exciting radiation is a function of the concentration of the solution. When the concentration remains constant, dosage is directly proportional to the time of exposure. Alteration of the concentration of the solution may greatly diminish the fluorescence or render it ineffective, in which case no enhanced effect might be obtained whatever the length of the exposure.

TABLE II.

(The percentage of survivors is calculated from the survivors in the non-irradiated phospho-tungstate control, which was assumed to be 100%.)

Organism.	No. per c.c.	Strength of phospho-tungstate.	X-ray dose in rads.*	Survival in saline after X-rays.	Survival in phospho-tungstate after X-rays.
B. coli	7,650	5%	$\frac{1}{4}$	40%	0·7%
,,	10,000	5%	$\frac{1}{8}$	32%	1·0%
,,	2,250	5%	$\frac{1}{4}$	55%	1·3%
S. aureus	15,200	5%	$\frac{1}{4}$	40%	4·3%
,,	12,400	5%	$\frac{1}{4}$†	32%	8·0%
,,	8,120	4%	$\frac{1}{4}$	38%	18·0%
B. coli	10,432	4%	$\frac{3}{16}$	40%	20·3%
,,	3,000	1%	$\frac{1}{8}$	39%	31·7%
,,	10,000	2%	$\frac{1}{8}$	41%	35·0%

* Source of rays: Broad focus Coolidge tube actuated by coil and mercury break; heating current, 4 amps.; primary circuit, 145 volts; spark-gap, 5 cm. (spheres of 5 cm. diameter); distance of cell from target, 34 cm.; 1 rad = 80 minutes' exposure.

† Softer X-rays; 3 cm. spark-gap.

IN VIVO EXPERIMENTS.

Calcium phospho-tungstate, like other soluble tungstates, precipitates protein, and would appear on the face of it to be eminently unsuitable for *in vivo* experiment. Nevertheless, it can be injected into the peritoneal cavity of a guinea-pig, provided the solution is not too strong.

1. *Toxicity Experiments.*

Injections of 2 c.c. of 1, 2, 3 and 4% solutions into the peritoneal cavities of guinea-pigs produced no apparent result; all the animals survived, and a post-mortem examination of the abdominal cavity after a week revealed no evidence of inflammation. With a 5% solution 3 c.c. proved fatal to a guinea-pig in 24 hours, the animal showing an intense inflammation of the peritoneum.

The injection of 2 c.c. of the same strength failed to kill the animal, but it was obvious that it felt distinctly unwell for 24 hours. Post-mortem a week later there was no abnormality. Subcutaneous injections of 2 c.c. of 5% solution were without effect.

2. *Fluorescence Experiments.*

Assuming that calcium phospho-tungstate emits ultra-violet rays when excited by X-rays, then these rays, if generated within the peritoneal cavity, would have a direct effect on the serous membrane itself. In order to determine whether the effect produced sufficient irritation to kill an animal, we first injected 2 c.c. of a 4% solution of calcium phospho-tungstate, anæsthetized the animal with urethane, and subjected its anterior abdominal wall to an X-ray exposure of 1 rad. Three animals so treated survived without any abnormal symptoms developing.

TABLE III.

Animal No.	Injected subcutaneously with—	X-ray dose.	Result.
1	0·5 c.c. of 5% calcium phospho-tungstate—staphylococcus emulsion	¼ rad	No immediate effect. Within 5 days small hard subcutaneous nodule appeared. In 10 days increased to size of cherry and appeared to contain small amount of pus. Completely resolved in 3 weeks. No breaking-down of tissue.
2	0·5 c.c. of 5% calcium phospho-tungstate—staphylococcus emulsion	*Nil*	Necrosis of skin over site of inoculation within 24 hours. Sloughing in 5 days. Recovered in 4 weeks.
3	0·5 c.c. of saline—staphylococcus emulsion	¼ rad	Necrosis of skin over site of inoculation within 48 hours. Subsequent necrosis of underlying tissues with perforation of peritoneal cavity and death in 10 days.
4	0·5 c.c. of saline—staphylococcus emulsion	*Nil*	Necrosis of skin over site of inoculation. Abscess formed and sloughing of tissues in 7 days. Recovered in 4 weeks.
5	0·5 c.c. of 5% calcium phospho-tungstate	¼ rad	No effect detected.
6	0·5 c.c. of 5% calcium phospho-tungstate	*Nil*	No effect detected.

3. *Bactericidal Experiments.*

In this series such guinea-pigs as were given an X-ray exposure were anæsthetized with urethane.

A 24-hour agar slope culture of a virulent hæmolytic *Staphylococcus aureus* was used. Inoculations were made subcutaneously in the anterior abdominal wall, about 4 square inches of the skin having previously been shaved.

The result is expressed in Table III, it being understood that where calcium phospho-tungstate and staphylococci were injected the living organism was emulsified in sterile 5% phospho-tungstate, using about 5 c.c. for one agar slope; where injections of saline and staphylococcus were injected the living organism was emulsified in sterile physiological saline in the same manner.

The result of this experiment demonstrated clearly that, by irradiating living staphylococci in the presence of 5% calcium phospho-tungstate, a definite bactericidal effect was obtained, though the small residual local lesion showed that sterilization was not quite complete.

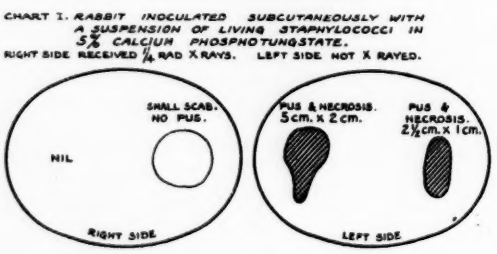
CHART I. RABBIT INOCULATED SUBCUTANEOUSLY WITH A SUSPENSION OF LIVING STAPHYLOCOCCI IN 5% CALCIUM PHOSPHOTUNGSTATE.
RIGHT SIDE RECEIVED 1/4 RAD X RAYS. LEFT SIDE NOT X RAYED.
SMALL SCAB. NO PUS.
NIL
RIGHT SIDE
PUS & NECROSIS. 5 cm. x 2 cm.
PUS & NECROSIS. 2½ cm. x 1 cm.
LEFT SIDE

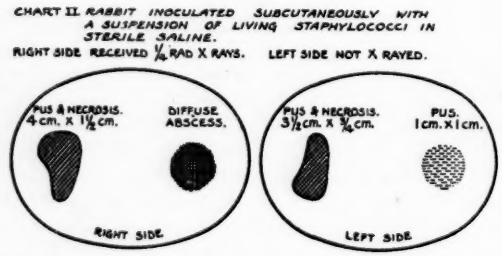
CHART II. RABBIT INOCULATED SUBCUTANEOUSLY WITH A SUSPENSION OF LIVING STAPHYLOCOCCI IN STERILE SALINE.
RIGHT SIDE RECEIVED 1/4 RAD X RAYS. LEFT SIDE NOT X RAYED.
PUS & NECROSIS. 4 cm. x 1½ cm.
DIFFUSE ABSCESS.
RIGHT SIDE
PUS & NECROSIS. 3½ cm. x ¾ cm.
PUS. 1 cm. x 1 cm.
LEFT SIDE

As a confirmatory experiment the procedure was repeated on two rabbits with some slight modifications, as under:

Suspensions of a virulent hæmolytic staphylococcus were made in sterile 5% calcium phospho-tungstate and in saline. The strengths of the suspensions were about half those used in the guinea-pig experiment, 10 c.c. of fluid being used to one agar slope of the organism. The skin on the flanks of the rabbits was shaved over an area of about 4 square inches.

Rabbit No. 1 received, in each of two positions in its right flank, a subcutaneous injection of 0·5 c.c. of the phospho-tungstate suspension. This flank was then given an X-ray exposure of ¼ rad. After the exposure the left flank was inoculated subcutaneously in each of two places with 0·5 c.c. of the phospho-tungstate suspension.

Rabbit No. 2 received in its right flank in two positions 0·5 c.c. of the saline suspension and was given a similar X-ray exposure, whilst the left flank was subsequently inoculated but not X-rayed.

By this procedure individual susceptibility was eliminated and a comparable result obtained.

The results are set out in Charts I and II. They comprise definite necrotic or purulent lesions in the animal receiving the saline suspension on both the irradiated and non-irradiated side, whilst the phospho-tungstate animal showed definite purulent or necrotic lesions on the non-irradiated side, with a complete negative in one of the irradiated sites, and only a small scab, with no pus-formation, on the other. At the last-named "site" a small hæmatoma developed when the inoculation was made, which was probably responsible for a certain amount of shielding. In spite of this no pus-formation occurred.

Experiments with intraperitoneal injections of a mixture of calcium phospho-tungstate and living organisms have not produced the same conclusive results, owing probably to the irritating action of the chemical allowing any surviving organisms to gain a foothold in the injured tissue.

These preliminary experiments appear conclusive enough to justify a claim that bactericidal fluorescence is a possible therapeutic measure, and that though calcium phospho-tungstate is far from being an ideal substance, yet its employment in superficial lesions may be found, by further experiment, to be useful and practicable.

CONCLUSIONS.

1. Bactericidal fluorescence is a possible means of developing effective physico-chemical therapy.

2. Calcium phospho-tungstate is a soluble substance emitting bactericidal wave-lengths when excited by X-rays, and can be used subcutaneously in animals for bactericidal purposes.

One of us (L. E. H. W.) is indebted to the Medical Research Council for a grant enabling him to carry out this work.

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ON WRIGHT'S CENTRIFUGE METHOD OF ESTIMATING PHAGOCYTOSIS AND THE RATE OF OPSONIZATION OF BACTERIA BY NORMAL SERUM.

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DURING the last twenty years much work has been done in connection with the opsonization of bacteria, but the question of the rate at which this process occurs has not been satisfactorily studied. In dealing with this problem there has been the difficulty that the union of the opsonic substance with the bacterium produces no change which can be measured except by estimating the amount of phagocytosis which takes place when opsonized bacteria are left in contact with leucocytes, and as the method hitherto used for inducing phagocytosis has been to incubate the mixture of leucocytes and opsonized bacteria (or it may be the mixture of leucocytes, serum and bacteria) for something like 15 minutes, this last period of incubation renders impossible any accurate estimation of the rate of union of the opsonic substance of the serum with the bacteria. This will be perfectly obvious when it is realized that if a mixture of washed leucocytes, serum and staphylococci are incubated together for 15 minutes, the resulting amount of phagocytosis is the same whether the staphylococci were previously opsonized or not.

In order, therefore, to investigate the rate of union of the opsonic substance with the bacteria, it was necessary to have a method whereby phagocytosis could be induced in a very short space of time. Such a method has recently been described by Sir Almroth Wright (1926). He has shown that if blood-corpuscles, serum and bacteria are mixed in a capillary tube and centrifuged at room temperature for from one half to one minute large numbers of the bacteria are taken up by the leucocytes. The appearance of the stained film when phagocytosis is induced by this method is exactly the same as when blood and bacteria have been incubated in the ordinary way. The bacteria appear to be inside the cells and in some cases there are vacuoles around them. It is quite obvious that in the conditions of the experiment—less than one minute contact of leucocytes and bacteria at room temperature—the leucocytes could not have moved about spontaneously and engulfed the bacteria, and it

would appear that the driving down of the particulate elements by the centrifuge brings the bacteria into contact with the leucocytes, when, if the bacteria have been opsonized, the leucocytes are able to ingest them, not by any process of pushing out large pseudopodia, but by some other and more rapid means.

Question as to whether Phagocytosis by the Centrifuge Method is Governed by the Same Conditions as Phagocytosis induced in the ordinary way.

Before using this rapid method or phagocytosis for any experimental work on opsonization, it was first necessary to inquire as to whether phagocytosis by this method was governed by the same conditions as phagocytosis by the ordinary method of incubation.

It was found that when bacteria and washed leucocytes were centrifuged together there was no phagocytosis of the bacteria, although there was ample phagocytosis when serum was present. Opsonization of the bacteria is therefore necessary. Heating the serum for one minute at 60° C. had the same result on phagocytosis by the centrifuged and the incubation methods, so that by neither method was there any appreciable amount of phagocytosis when heated serum was used.

The effect of dilution of the serum was investigated, and, as will be seen from the following experiments, there is a very close correlation between the results obtained with the centrifuged and the incubation methods.

Experiment 1.—Equal volumes of serum (or diluted serum), washed corpuscles and staphylococci were mixed. One set of these mixtures was incubated for 15 minutes at 37° C. and the other set were centrifuged for 1 minute. Films were made and the amount of phagocytosis estimated in the usual way with the following result:

Dilution of serum.	Amount of phagocytosis.			
	By centrifuge method.		By incubation method.	
	Bacteria per leucocyte.	Index.	Bacteria per leucocyte.	Index.
Undiluted	4·27	1·00	2·63	1·00
1 in 2	4·22	0·99	2·41	0·93
1 in 4	3·73	0·88	2·12	0·81
1 in 8	3·37	0·79	1·80	0·69
1 in 16	2·86	0·66	1·72	0·66

Different concentrations of bacterial suspension were used in the two series, so the actual numbers of the bacteria ingested are not comparable, but when the opsonic index is worked out, taking the undiluted serum in each case as unity, the close correlation between the two methods will be seen.

Experiment 2.—Equal volumes of serum (or diluted serum) and staphylococcal suspension were allowed to remain in contact at 37° C. for 5 minutes or 18° C. for 15 minutes, when to two volumes of the mixture was added one volume of washed corpuscles, and the mixtures were centrifuged for one minute at room temperature. The amount of phagocytosis was compared with that obtained by incubation of equal volumes of washed corpuscles, serum and staphylococcal suspension for 5 and 15 minutes at 37° C. In this case the same staphylococcal suspension was used in all cases, so the amount of phagocytosis in each case is comparable. The results obtained were as follows:

Dilution of serum.	Incubated 15 minutes at 37° C.		Incubated 5 minutes at 37° C.		Opsonized 5 minutes at 37° C., then centrifuged.		Opsonized 15 minutes at 18° C., then centrifuged.	
	Cocci per 100 leucocytes.	Index.	Cocci per 100 leucocytes.	Index.	Cocci per 100 leucocytes.	Index.	Cocci per 100 leucocytes.	Index.
Undiluted	571	1·00	321	1·00	468	1·00	423	1·00
1 in 10	368	0·64	196	0·60	360	0·75	251	0·60
1 in 20	277	0·44	127	0·39	254	0·54	224	0·54
1 in 30	207	0·32	77	0·24	178	0·40	130	0·31
1 in 40	138	0·28	69	0·21	116	0·25	126	0·30
1 in 50	92	0·14	51	0·16	61	0·13	59	0·14

These observations show that phagocytosis induced by the centrifuge method is dependent on the same factors as is phagocytosis observed after incubation in the ordinary way, so it becomes possible to use the rapid centrifuge method as a substitute for the slow incubation method in experimental work on opsonization and phagocytosis.

Effect of Varying the Time of Centrifuging on the Amount of Phagocytosis.

It has been seen from the experiments quoted above that considerable phagocytosis takes place during 1 minute's centrifuging. Further experiments were carried out to see whether increasing the time of centrifuging made much difference to the amount of phagocytosis.

Equal volumes of corpuscles, serum and staphylococcal suspension were mixed and immediately centrifuged for various lengths of time, after which the amount of phagocytosis was estimated. Another series of estimations was done where the serum and staphylococci had been in contact for 15 minutes at 37° C. (a time which, as we shall see later, is more than enough to ensure complete opsonization of the bacteria) before the corpuscles were added and the mixtures centrifuged. The results obtained were as follows:

Time of centrifuging.	Number of cocci ingested by 100 leucocytes.	
	Bacteria previously opsonized.	Bacteria not previously opsonized.
½ minute	124	20
1 „	177	57
2 minutes	173	97
4 „	193	127

These results have been confirmed in a number of experiments where different concentrations of bacterial suspension have been used giving counts up to 10 cocci per cell, and it seems clear that when previously opsonized bacteria are used phagocytosis is complete after centrifuging for 1 minute. With "raw" or non-opsonized bacteria, however, there is a gradual increase in the number ingested as the time of centrifuging is increased, and even after 4 minutes phagocytosis is far from complete. This is, as we shall see later, in all probability due to the slow rate of opsonization at room temperature.

Having thus determined that with fully opsonized bacteria phagocytosis is complete in one minute, this was the time chosen in the experiments now to be described.

Influence of Temperature on Phagocytosis by the Centrifuge Method.

As the process of centrifugalization at incubator temperature was troublesome, it was necessary to determine whether there was much difference in the rate of phagocytosis at 37°C. and 18° C. Ledingham (1908), in a valuable contribution to the study of phagocytosis, has shown that by the incubator method there is comparatively little difference in the rate of phagocytosis at these temperatures, and an experiment was undertaken to ascertain whether this held good also for the centrifuge method.

Serum and staphylococci were mixed and incubated for 20 minutes at 37° C. so that the cocci should be fully opsonized. Washed corpuscles were added, and the mixtures were immediately centrifuged in water at various temperatures. The temperature of the water in the buckets, of course, approached room temperature during the process of centrifuging, but as soon as was practicable (about 4 minutes after centrifuging was complete) the temperature in the bucket was taken, so that an approximation of the temperature at which the centrifuging had been carried out could be obtained.

The results obtained are shown in Table I.

TABLE I.—*Influence of Temperature on Phagocytosis by the Centrifuge Method.*

Temperature of centrifuge bucket (° C.)		Probable temperature during experiment.	Cocci ingested by 100 leucocytes.
At commencement.	After centrifuging completed.		
0	0	0	63
16	16	16	260
37	23	32	353
—	4	4	100
16	16	16	293
37	24	32	370
40	25	34	363

These experiments show that while there is a very considerable diminution in the amount of phagocytosis at very low temperatures, there is comparatively little difference in the numbers of cocci ingested at room and incubator temperatures, which is in agreement with Ledingham's results, already referred to, obtained by the incubation method.

This finding is of importance, as it made it unnecessary to take special precautions about the temperature at which the centrifugalization was carried out, so in all cases in the experiments to be described it was done at room temperature.

Rate of Opsonization of Bacteria by Normal Serum.

As a result of the preliminary experiments quoted above, it was possible to arrive at the rate of opsonization by leaving serum and bacteria in contact for various times, and then adding leucocytes and centrifuging the mixture and noting the amount of phagocytosis which had occurred.

Equal volumes of serum and a suitable suspension of staphylococci were mixed and incubated at 37° C. At various intervals two volumes of the mixture were added to one volume of washed corpuscles and the whole centrifuged for one minute, when the amount of

phagocytosis was estimated in the usual manner. The same thing was done, using suspensions of *B. coli* and the tubercle bacillus instead of staphylococcus. The results obtained are shown in the following table:

TABLE II.—*Rate of Opsonization of Bacteria at 37° C.*

Staphylococcus.		Tubercle bacillus.		*B. coli.*	
Time of opsonization.	Bacteria per 100 leucocytes.	Time of opsonization.	Bacteria per 100 leucocytes.	Time of opsonization.	Bacteria per 100 leucocytes.
1 minute	256	1 minute	160	1 minute	22
3 minutes	508	3 minutes	262	3 minutes	148
6 ,,	570	5 ,,	258	5 ,,	198
8 ,,	600	10 ,,	210	10 ,,	240
11 ,,	607	15 ,,	238	15 ,,	236
16 ,,	620	30 ,,	236	30 ,,	252
42 ,,	631				

It will be seen that with all these microbes the opsonization is complete in a few minutes, but with the shorter periods of incubation there would seem to be definitely less opsonization in the case of *B. coli* than with the tubercle bacillus or the staphylococcus. At a temperature of 37°C., however, the whole process is so rapid that it is difficult to make out the differences clearly, so a fresh series of experiments was undertaken, but this time the process of opsonization was carried out at 18° C. Ledingham (1908) has shown that at this temperature the process of opsonization is much slower than it is at 37°C., and it was hoped that by lengthening the time necessary for opsonization it could easily be seen whether there was any real difference in the rate of opsonization of these different bacteria. The method of experimentation was exactly the same as in the previous series, except that the serum-bacteria mixtures were incubated at 18°C. for various intervals before the washed corpuscles were added and the mixtures centrifuged. The results obtained appear in Table III.

TABLE III.—*Rate of Opsonization of Different Bacteria by Normal Serum at 18° C.*

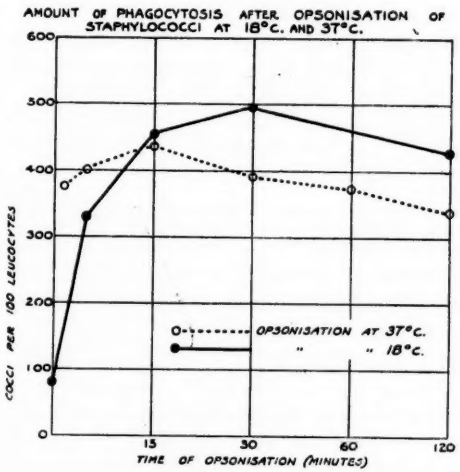
Time of opsonization at 18°C. before washed corpuscles added.	Number of bacteria ingested by 100 leucocytes.		
	Staphylococci.	Tubercle bacilli.	*B. coli.*
0 (centrifuged immediately)	51	70	1
5 minutes	328	134	1
10 ,,	500	304	2
15 ,,	474	336	22
30 ,,	556	484	116
60 ,,	504	572	245
120 ,,	—	814	366
240 ,,	—	—	346
Control. (Bacteria fully opsonized by incubation with serum for 15 minutes at 37°C.)	438	758	303

This series of observations brings out two interesting points: firstly that the rate of opsonization of different bacteria varies very considerably, and secondly, that the bacteria are apparently more fully opsonized at 18°C. than they are at 37°C.

Of the three bacteria examined, opsonization of the staphylococcus proceeds most rapidly, so that it is complete in 10 minutes at 18°C. With tubercle bacilli after the same time there is about 40 per cent. of the amount of phagocytosis which obtains when opsonization is complete, while with *B. coli* after 10 minutes' opsonization there is no phagocytosis.

With both tubercle bacilli and *B. coli* opsonization was incomplete after 1 hour, but was complete after 2 hours.

The second point, the apparent increase in the total opsonization at 18°C., was very unexpected. To illustrate this point the figures of an experiment in which the rate of opsonization of staphylococci by a normal serum was tested at 37° and 18°C. are plotted into curves which are superposed. From this chart it will be seen at a glance that while there is some delay in the rate of opsonization at the lower temperature, yet ultimately the amount of phagocytosis rises to a higher level.



This was observed on several occasions, but to make sure that it was of regular occurrence and not due to any chance errors, the following experiment was carried out:

Serum was obtained from six healthy laboratory workers and this was mixed with an equal volume of a staphylococcus suspension of suitable strength. Each of these serum-staphylococcus mixtures was divided into two parts, one of which was incubated at 37° C. and the other at 18°C. for one hour. One volume of washed blood-corpuscles was then mixed with two volumes of the serum-staphylococcus mixture and centrifuged for one minute, when the amount of phagocytosis was estimated. The results are given in Table IV.

TABLE IV.—*Amount of Phagocytosis Observed after Opsonization at 18° C. and 37° C.*

Serum.	Number of cocci ingested by 100 leucocytes.	
	Opsonized at 18°C.	Opsonized at 37°C.
A. F—	870	520
R. M. F—	788	664
A. F. H—	750	722
L. C—	706	580
L. M—	806	656
R. H—	876	752

It will be seen that in every case there was more phagocytosis when opsonization had been carried out at 18°C. The difference varied with the different serums, and in one case the increase amounted to nearly 70 per cent. This serum was tested on two other occasions, and the increase on the amount of phagocytosis when opsonization had been carried out at the lower temperature was in the one case 75 per cent. and in the other 95 per cent. In these experiments the results were the same whether living or dead staphylococci were used.

Although at first sight it would appear from these results that opsonization of bacteria is more complete at 18° C. than it is at 37°C., such a result was so surprising that some other explanation was sought for. If the opsonization was actually more complete at the lower temperature, then presumably more of the opsonic substance would be bound to the microbes and less would be left in the serum. An experiment was made to see if this were so.

Equal volumes of serum and staphylococcus suspension were mixed and incubated at 37° C. and 18° C. After 1 hour the staphylococci were centrifuged out and the opsonic power of the supernatant fluid tested, when it was found that the serum which had been absorbed at 37° C. had apparently less opsonic power than that absorbed at 18° C.:

Serum absorbed at 37° C. Number of cocci per 100 leucocytes = 78
„ „ 18° C. „ „ = 127

It would appear, therefore, that more of the opsonic substance is not joined up to the bacterium at the lower temperature, and a more likely explanation of the difference in the amount of phagocytosis when opsonization is carried out for a considerable time at 37° and 18° C. is that during prolonged incubation at the higher temperature some substance was set free into the serum which had a toxic effect on leucocytes with consequent partial inhibition of their phagocytic power.

That such a toxic substance was liberated was confirmed by the following experiment:

A mixture of equal volumes of serum and a staphylococcus suspension was made, and one-half of this was incubated for 1 hour at 37° C. and the other for the same time at 18° C. Then one volume of washed corpuscles was added to two volumes of each of these mixtures, centrifuged for 1 minute and the resulting phagocytosis estimated. The results were as follows:

Opsonized at 18° C. . . . 872 cocci per 100 leucocytes.
„ 37° C. . . 654 „ „

The remaining portions of the serum-staphylococcus mixtures were then centrifuged to remove the cocci, and one volume of each of the supernatant fluids was added to one volume of washed corpuscles, serum and staphylococci, mixed and incubated for 15 minutes at 37° C., when the amount of phagocytosis was estimated with the following result:

Mixture containing supernatant from serum-staphylococcus mixture incubated at 18° C.	108 cocci per 100 leucocytes.
Mixture containing supernatant from serum-staphylococcus mixture incubated at 37° C.	60 „ „

This experiment shows that when serum and staphylococci are incubated at 37° C., the serum acquires the property of inhibiting to some extent the ingesting of bacteria in an ordinary phagocytic mixture. This inhibitory substance is not evident in the serum when incubation is carried out at 18° C. for one hour, but when opsonization of staphylococci is prolonged for 3 or 4 hours at 18° C. there is sometimes seen to be a decided drop in the amount of phagocytosis, which is probably due to the liberation of the same toxic substance which is evident in the serum after one hour at 37° C.

SUMMARY.

Wright's method of inducing phagocytosis by centrifuging for a short time is governed by the same conditions as is phagocytosis by the ordinary methods.

Working with fully opsonized bacteria phagocytosis is complete after centrifuging for one minute at room temperature.

There is comparatively little difference in the rate of phagocytosis at room or incubator temperature.

The opsonization of bacteria at 37° C. is very rapid, being complete in a few minutes. At room temperature the rate is much slower.

There is considerable difference in the rate of opsonization of different bacteria.

After prolonged incubation of serum and staphylococci a toxic substance is liberated in the serum which inhibits phagocytosis to some extent.

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THE INHIBITION OF INSULIN ACTION BY TOXÆMIAS AND ITS EXPLANATION.—I: THE EFFECT OF DIPHTHERIA TOXIN ON BLOOD-SUGAR AND INSULIN ACTION IN RABBITS.*

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THE effect of toxæmias and different forms of sepsis in inhibiting insulin in diabetes is one of the chief puzzles of insulin action and the greatest weakness of insulin therapy. All workers are agreed that sepsis and infections reduce the diabetic's carbohydrate tolerance, are the commonest immediate cause of coma, necessitate a large increase of the dose in insulin cases, and sometimes inhibit altogether the hypoglycæmic action of huge doses of insulin. The problem of this inhibition is therefore of great physiological interest and therapeutic importance.

It is obvious that the failure of insulin, either relative or absolute, induced by sepsis in diabetics might be due (1) to a failure to produce the usual amount of endogenous insulin, although this would be only a partial explanation where large doses of injected insulin failed to act altogether; (2) to a direct chemical effect of the toxins on insulin; or (3) to the production of a metabolic condition which will not respond to insulin in the usual way. The third was thought to be the most likely explanation, and the simplest way to attack the problem seemed to be to ascertain whether a similar insulin-resistant condition could be produced in normal animals. Rabbits were chosen for the purpose, and the plan of the investigation was to observe in each animal its blood-sugar response to insulin before and after the injection of different infective and toxæmic agents. A variety of these have been and are being tried with variable success, and so far the clearest results have been obtained with diphtheria toxin. With this, however, a condition has been produced in normal rabbits in which the blood-sugar is spontaneously raised, and in which the power of insulin to reduce blood-sugar is greatly diminished and sometimes entirely absent. These experiments are described in this paper; their significance is discussed, and it is suggested that they afford a solution to the problem.

METHODS.

The rabbits used were of various breeds and mostly weighed between 2 and 2·5 kgm. They were fed on oats and bran mixture with an abundance of cabbage. When the experiments were commenced about 10 a.m. they had been without food for 17 to 20 hours. They ate very little by the

* A Report to the Medical Research Council.

third and fourth day after the injection of diphtheria toxin, and, after that, not at all.

The blood was collected from the heart with a fine hypodermic needle and syringe in every case; only in rabbit 9 was any serious hæmopericardium found, and even this did not seem to interfere with the duration of life. This method of collection is very quick and easy, and was originally adopted owing to the difficulty of getting blood from the ear veins after the injection of ergotamine in moribund rabbits. The estimations were carried out in duplicate on oxalated whole blood by Maclean's method.

The diphtheria toxin was supplied by the kindness of Dr. Hartley, of the National Institute for Medical Research, and was given into the subcutaneous tissues of the abdominal wall, where it produced the usual typical sore. The doses varied between 0·001 c.c. and 0·025 c.c. per kgm. of rabbit weight. The rabbits reacted very differently, but a dose of 0·004 c.c. was the most generally successful in causing a slow death of the rabbits after 4–7 days. When the first dose was ineffective and a second had been given death usually occurred within 36 hours, even although the first dose had had no apparent effect.

The plan of the investigation is obvious from the tables of the results. The effect of a small dose of insulin was followed for a few hours on the blood-sugar. The toxin was then given and the result of a similar dose of insulin was followed every day until the death of the animal. The rectal temperature was closely watched both as an indication of reaction and of impending collapse and death. The dose of insulin was small—usually one clinical unit—because it was felt that large doses might obscure any slight effect that the toxin might have on insulin action. As a matter of fact, the fall in blood-sugar caused by one unit was no greater than by half a unit. The same dose of insulin was used throughout the investigation of any one rabbit.

THE RESULTS.

Selected examples of the results obtained are given in the tables. No contradictory results have been obtained in our other experiments. In the tables the blood-sugar concentration is expressed as milligrammes per cent., the temperature as degrees Fahrenheit, and the figures within brackets show the maximum drop in blood-sugar after insulin on each day. The toxin was injected in the evening of the control-curve day, and the curves on the succeeding days are placed alongside for comparison. The results have been grouped as far as possible to show separate phenomena, which have been divided into the three following groups:

Insulin action reduced or absent (Tables A, B, C, D).—This is seen in rabbits 1, 6, 7, 8 and 15. It will be seen that after the toxin the absolute fall in blood-sugar is always less than on the control day and never reaches such a low level. The initial fasting blood-sugar is seen to vary greatly, and it is mostly on days when the fasting level is low that insulin action is diminished or absent. In rabbits 8, 15 and 3 insulin seemed to stimulate a temporary rise in blood-sugar. When the fasting blood-sugar is raised insulin always acts to a certain extent and brings the blood-sugar down to a low

Tables A, B, C, D, Showing Insulin Action Reduced or Absent.

TABLE A.

Rabbit 1.	Control.			1st day.		2nd day.	
Blood-sugar before insulin	121	101°F.	0·01 c.c./kgm. diphtheria toxin.	109	104·7°	93	99·6°
½ hour after 1 unit	62			100		91	
1 ,, ,,	67			86		85	
2 hours ,,	64			54		78	
3 ,, ,,	73			—		Died at night.	
	(57)			(55)		(15)	

TABLE B.

Rabbit 6.	Control.		1st day.	2nd day.	3rd day.	6th day.		7th day.	
	102°F.	0·003 c.c./kgm. diphtheria toxin.	103·3°	103·7°	103°			*180	97·8°
Blood-sugar before insulin	126		140	118	122	154	99·4°	223	97·2°
½ hour after ½ unit.	—		127	118	124	—	—	—	—
1 ,, ,,	84		108	111	121	121	99·2°	251	97·0°
2 hours ,,	73		102	111	119	111	98·9°	214	96·4°
3 ,, ,,	—		—	108	109	103	98·8°	201	96·0°
4 ,, ,,	—		—	—	—	111	98·6°	160	95·4°
5 ,, ,,	—		—	—	—	—	—	268	—
6 ,, ,,	—		—	—	—	138	98·4°	Died.	
11 ,, ,,	—		—	—	—	155	97·8	—	—
	(53)		(38)	(10)	(13)				

* Blood-sugar level 1 hour previous to the following figure.

TABLE C.

Rabbit 7.	Control.		1st day.	2nd day.	3rd day.	4th day.	5th day.
	101·8°F.	0·003 c.c./kgm. diphtheria toxin.	102·7°	103·4°	103·4°	101·9°	101·2°
Blood-sugar before insulin	117		131	118	138	108	134
½ hour after ½ unit	—		—	115	111	112	129
1 ,, ,,	53		118	109	82	116	97
2 hours ,,	42		121	111	76	114	62
4 ,, ,,	—		139	—	—	109	—
	(75)		(10)	(7)	(62)	(+6)	(72)
							Died 8th day.

Rabbit 8.	Control.		1st day.	2nd day.	3rd day.	4th day.	5th day.
	100·8°F.	0·004 c.c./kgm. diphtheria toxin.	105°	103·2°	102°	103°	103·4°
Blood-sugar before insulin	125		118	151	118	109	125
1 hour after 1 unit	68		84	127	127	110	103
2 hours ,,	83		80	104	130	109	113
3 ,, ,,	—		84	99	132	—	—
4 ,, ,,	—		—	—	126	117	120*
4½ ,, ,,	—		—	—	—	—	192
5¾ ,, ,,	—		—	—	—	—	305
	(57)		(38)	(52)	(+14)	(0)	(22)
							Died 7th day.

* 0·5 mgm. adrenalin.

TABLE D.

Rabbit 15.	Control. 101·4°F.		1st day. 103·4°	2nd day. 102·4°	3rd day. 100·2°	4th day. 175 94·0°
Blood-sugar before insulin	123	0·004 c.c./kgm. diphtheria toxin.	112	118	105	175
1 hour after 1 unit	85		86	85	127	Died.
2 hours ,,	96		81	85	118	
3 ,, ,,	75		113	90	106	
	(48)		(31)	(33)	(+22)	

normal, but not to a hypoglycæmic level. In the prelethal rise (see later) insulin seemed to act in rabbit 6 (7th day) in reducing the blood-sugar for a time. Its action on that day is similar in its time effect to its action on a diabetic and not on a normal. In the diabetic it produces a slow lowering of the blood-sugar, maximal in the third or fourth hour, whereas in a normal (both rabbits and men) the drop is greatest in the first hour, and recovery usually takes place in the second and third hours after small doses. This delayed fall in blood-sugar is seen in most of the tables on comparing the control with subsequent days. Insulin seems to act better during the febrile reaction to the toxin than when the fever has worn off. This was very clear in other experiments not recorded.

Prelethal rise in blood-sugar and its control by ergotamine (Tables E, F, G).—This is seen clearly in rabbits 3, 7, 8 and 19, and occurred in every rabbit in which a slow death after 3–10 days was observed. It did not occur in the more rapid deaths, nor in the rapid death that followed a second dose of the toxin after the failure of the first. Ergotamine was found to control this prelethal rise and to reduce the blood-sugar to normal (femergin, the preparation used, contains 1 mgm. of ergotamine tartrate in 1 c.c.). The effect of femergin is a little obscured in rabbit 8 by the injection of infundin.

TABLE E.—*Prelethal Rise of Blood-Sugar.*

Rabbit 3.		1st day.		2nd day.		3rd day.	
Blood-sugar before insulin		139	105·5°	108	102°	122	101·7°
½ hour after ½ unit		140	—	128	—	—	—
¾ ,, ,,		—	—	163	100·6°	142	—
1 ,, ,,		123	—	163	101·7°	—	—
2 hours ,,		104	—	—	—	144	100·2°
3 ,, ,,		—	—	—	—	196	—
4 ,, ,,		—	—	—	—	218	—
5 ,, ,,		—	—	—	—	210	99·2°
6 ,, ,,		—	—	—	103·2°	259	—
7 ,, ,,		Ate very little.		Ate nothing.		—	97·0°
8½ ,, ,,	0·003 c.c./kgm. diphtheria toxin.	—	—	—	—	Died.	
		(35)		(+55)		(+22)	

Although the temperature was beginning to fall, the blood-sugar (129) did not suggest that the prelethal rise was about to commence as the second blood-sugar showed (157), and it was desired to try the effect of pituitary extract in

this condition. It will be seen that infundin gave a much greater rise of blood-sugar than in a normal rabbit. Adrenalin, too, gave a marked hyperglycæmia in rabbit 8, and both may probably be taken as evidence that, in the metabolic condition produced by the toxin, glycogenolytic agents cause an increased reaction. It seemed also that femergin prolonged the life of the moribund rabbits. Indeed, it is tempting to attribute the recovery of rabbit 19 directly to femergin, for no other rabbit with a temperature of 99·4° F. and a rising blood-sugar recovered.

TABLE F.—*Control of Prelethal Rise of Blood-Sugar by Femergin.*

Rabbit 7.—8th day.			Rabbit 8.		
Time.	Blood-sugar.	Temperature.	Time.	Blood-sugar.	Temperature.
10.0 a.m.	229	96·8°	10.0 a.m.	129	100·0°
11.0	235	—	11.0	157	99·1°
11.15	2 c.c. femergin.		11.15	1 c.c. infundin.	
11.45	151	95·0°	12.0	227	96·2°
12.15 p.m.	182	—	1.0 p.m.	172	—
12.45	154	—	1.10	2 c.c. femergin.	
1.15	114	—	2.0	159	96·0
2.0	104	94·6°	2.45	142	95·8°
3.35	115	—	3.35	135	—
3.40	2 c.c. femergin.		4.35	136	96·8°
4.10	102	—	5.20	132	97·0°
4.45	110	—	6.20	—	97·3°
6.0	104	—	11.15	125	98·4°
8.15	105	94·4°	Died during the night.		
	Died.				

TABLE G.—*Control of Prelethal Rise of Blood-Sugar by Femergin.*

Rabbit 19.	6th day after 0·004 c.c./kgm.		Remarks.
	Blood-sugar.	Temperature.	
9.45 a.m.	157	99·4°	
10.25	176	—	
11.10	170	—	
	Looked limp.		
11.15	2 c.c. femergin.		Recovered completely. Started eating again on 13th day. Had a very small dose of 0·001 c.c./kgm. and died suddenly 3 days later.
11.55	154	100°	
12.40 p.m.	156	—	
1.25	123	100·6°	
2.10	107	—	
2.55	119	99·4°	
4.0	96	99·2°	
9.30	—	100·7°	
	Looked stronger.		
11.20	116	101·2°	
	Looked stronger.		

Relation of body temperature to blood-sugar (Table H).—A curious rise in blood-sugar accompanied by a fall in temperature occurred in rabbits 9 and 14. This has not been recorded by other workers as far as we know, and, when it first occurred in rabbit 9, was thought to be an error in the blood-sugar estimation, although the source of error could not be seen. Unfortunately the estimations were not carried out till late in the afternoon, so that the blood-sugar was not estimated at the fifth hour. Rabbit 9 had received no injection of toxin, and rabbit 14, although it had been injected with toxin 8 days previously, seemed to be unaffected by it. The phenomenon, therefore, does not seem to be connected with the action of diphtheria toxin.*

TABLE H.—*Relation of Body Temperature to Blood-Sugar.*

Rabbit 9.	Blood-sugar.	Temperature.
Before insulin	139	102°
1 hour after 1 unit	75	102°
2 hours ,,	86	101·4°
3 ,, ,,	412	97·6°
5 ,, ,,	—	101°

Rabbit 14.	Blood-sugar.	Temperature.	Remarks.
Before insulin	128	102·8°	—
1 hour after 1 unit	71	103·0°	—
2 hours ,,	207	100·1°	Limp
3 ,, ,,	129	101·7°	Recovering
4 ,, ,,	137	102·4°	—

HISTOLOGICAL CHANGES IN THE THYROIDS AND ADRENALS OF THESE RABBITS.

The organs of some of these rabbits were examined histologically and showed marked abnormalities. Besides cloudy swelling and fatty changes in the liver and kidneys, the thyroids and adrenals were greatly changed. Unfortunately, although the importance of examining these latter organs was recognized from the first, it was not realized that they were to prove so important, and no special fixing and staining methods were used to show chromaffin, etc. But ordinary histological methods have shown the usual marked changes that occur in these organs after diphtheria toxin (see later), and Dr. Cramer, of the Imperial Cancer Research Fund, has been kind enough to verify them in these rabbits. Rabbit 6, for example, showed great congestion of the thyroid (which was visible even to the naked eye), disappearance of the colloid, frequent necrosis of the alveolar cells and destruction and confluence of many of the alveoli. The adrenal showed partial disappearance of the lipoid, and rabbits 5 and 8 showed complete disappearance

* The same phenomenon has since been observed in three other rabbits.

of the lipoid. Further investigations are being undertaken with special histological methods, but it is quite clear that the toxin has produced exhaustion and marked degeneration in these organs—conditions which are usually preceded at some stage by stimulation and over-activity.

It would seem best to postpone the discussion of these results, as it is impossible to present clearly the explanation of them which we have to offer without first referring to a number of closely associated factors. It will no doubt already have occurred to many, as it did to us early in the investigation, that the changes in the thyroid and the adrenal might prove the explanation of the problem, and this indeed is the one which we have to offer. But we are in the meantime to refer to certain substances which are known to antagonize insulin physiologically and to see what evidence there is that toxæmias stimulate these antagonistic influences.

OTHER SUBSTANCES ANTAGONISTIC TO INSULIN.

The extracts of the adrenal, thyroid and pituitary gland are known to be physiological antagonists to insulin. It is with the first two that we are concerned here, because the evidence about their mode of action is much clearer and better understood than in the case of pituitary extract.

The action of insulin in lowering blood-sugar can be entirely prevented by the simultaneous administration of appropriate doses of *adrenaline*. Further, rabbits can be divided into two types according to whether they are most sensitive to adrenaline or insulin. Those which respond with a high hyperglycæmia to a dose of adrenaline, presumably because their sympathetic nervous system is easily stimulated, are resistant to insulin, and those which are resistant to adrenaline are much more sensitive to insulin (Burn and Marks, 1925). The hyperglycæmic effect of adrenaline is known to be due to glycogenolysis, by which it mobilizes blood-sugar from the glycogen in the liver and perhaps in the muscles. Ergotamine, which paralyses the sympathetic to the liver, prevents this adrenaline glycogenolysis. But when the liver is already glycogen-free, Pollak (1909) and others have shown that adrenaline increases the amount of glycogen in the liver—glycogen which must have come indirectly from protein and perhaps from fat. Adrenaline therefore raises blood-sugar by mobilization of glycogen, and also produces new sugar from other substances which can apparently be stored as glycogen, presumably when the glycogenolytic effect of the adrenaline has worn off.

Thyroid extract also affects carbohydrate metabolism and the disturbance in Graves's disease is well recognized. Thyroid feeding in rabbits has been shown by Burn and Marks (1925) to make them at first very resistant to insulin, and afterwards increasingly sensitive to it when the thyroid feeding has emptied more and more glycogen out of the liver. It is very probable that this is due to the effect of the thyroid on the adrenal, which it first stimulates to over-activity and then finally exhausts (Cramer, 1926).

We have, therefore, in the thyroid and the adrenal a mechanism which can antagonize insulin action, and if there is evidence that toxæmias stimulate the activity of these two glands, we should have an explanation to the effect of toxæmias in inhibiting insulin action. We shall now consider this evidence.

THE EFFECT OF BACTERIAL INFECTIONS ON THE ADRENAL AND THYROID.

We have already mentioned the striking histological changes that were present in these rabbits poisoned with diphtheria toxin, and, on searching the literature on the subject, we have found many records of changes in these glands as the result of different toxæmias and infections. Cramer (1926) has recently published a paper on the effects of fever and infections on the thyroid-adrenal "apparatus," as he terms the mechanism, and readers are referred to this paper for a general review of the literature on the subject.

Adrenal.—Profound changes are found in the adrenals in many forms of experimental infections in animals, in particular with diphtheria toxin and *B. Welchii*, but also with pneumococci, streptococci, staphylococci and other organisms. The effects have been chiefly studied histologically by the content of the cortical lipoid and the medullary chromaffin. The effects observed vary with the virulence, dose and nature of the infective or toxæmic agent. With small doses the gland is stimulated to over-activity, with large and rapidly toxic doses the gland is rapidly exhausted and loses all its lipoid and chromaffin. Cramer describes these two effects as "adrenalotonic" and "adrenalotoxic."

Evidence of the same condition of the adrenal has been obtained frequently in human pathology in very varied infections—measles, scarlatina, tuberculosis, etc.—although it is rare to obtain the specimens sufficiently fresh to give very clear histological pictures. Goldzieher states that congestion with or without hæmorrhages, thrombosis and necrosis, etc., are of *constant* occurrence in septic infections in contrast with the rarity of such changes in other organs.

The relation of these adrenal changes with variations in the blood-sugar picture never seems to have been pointed out, but we shall attempt to show this relation in the discussion of the results obtained in our rabbit experiments.

The thyroid gland.—The thyroid has also been shown by many observers to show changes in bacterial infections (consult Cramer's paper for references), mostly of the nature of hyperæmia and disappearance of the colloid, both of which changes were present in the thyroids of our rabbits to a marked degree. This occurs both experimentally in animals and in human diseases. Scarlet fever, typhoid and acute tuberculosis are said to be particularly powerful in stimulating the thyroid. In clinical medicine it is well known how often a severe infection—influenza, typhoid or septic tonsillitis—seems to be the direct cause of the onset of Graves's disease—a permanent over-activity of the thyroid gland. Or, again, myxœdema and Addison's disease have been known to commence from similar acute infections, probably because the endocrine glands concerned have, in these cases, been exhausted by the infection instead of over-stimulated.

The evidence, therefore, would seem complete that bacterial infections stimulate the thyroid-adrenal apparatus to increased activity and perhaps ultimately exhaust it, and in this we see a mechanism which would antagonize the usual effect of insulin and explain its failure to act in diabetes during infections. We shall see later that the same upset of carbohydrate metabolism and disturbance of the blood-sugar picture is seen to occur in infections in normal animals and human beings, although the disturbance is much less obvious than in diabetics. In the meantime let us see how this hypothesis of

a stimulated, sometimes exhausted, thyro-adrenal apparatus fits in with the blood-sugar picture and the different facts recorded in these tables, remembering, as a starting-point, that these rabbits showed very definite changes in these two glands.

DISCUSSION OF RESULTS.

Variability of the blood-sugar.—It is clear from the blood-sugars recorded in the tables, as well as from others not included, that there is a great tendency for the fasting blood-sugar to vary from time to time, the variations being from 90 to 150 mgm. per cent. As these rabbits mostly refused all food after the second or third day, and since by this time the glycogen store of a rabbit is nearly exhausted, it is clear that these variations in fasting blood-sugar must be due to variations in the rate of production of sugar from endogenous sources, *i. e.* from protein and perhaps from fat. Either, when the blood-sugar is high, an excessive production of sugar is taking place direct from other substances, or a mobilization of whatever glycogen has been temporarily stored from this new sugar is suddenly taking place. It is probable that new-formed sugar from other substances does pass through the form of glycogen, because a liver that has been deprived of glycogen by starvation or other methods gradually recovers a certain content of glycogen as starvation proceeds. Injections of adrenaline increase the glycogen content of the glycogen-free liver presumably because it stimulates the new production of sugar from protein and fat, which, if not immediately wanted for burning (Pollak, 1909), becomes stored as glycogen when the adrenaline action wears off. It is interesting to note that certain bacterial emulsions increase the glycogen content of the liver and muscles (Hirsch and Rolly, 1903)—a fact for which these authors gave no explanation, but which we would explain by the effect of these bacterial emulsions in stimulating the adrenal in its production of new sugar. It is unlikely that the blood-sugar variations are dependent on the different rate of sugar used in metabolism, because these rabbits crouched without moving at the bottom of their cages during the whole day and refused all food. The variations would appear to be due to the varying activity of the adrenal or thyroid apparatus, which we must imagine would vary between periods of over-activity and relative exhaustion. Therefore a new production of sugar would be supplied at different rates at different times, whether this sugar comes from temporarily stored glycogen or direct from protein or fat. This will be discussed further in relation to the failure of insulin action.

Prelethal rise in blood-sugar controlled by ergotamine.—Ergotamine, which paralyses the mobilization of sugar from the liver by sympathetic impulses and adrenalin, stops the prelethal rise in blood-sugar in these rabbits. It seems clear, therefore, that the prelethal rise is due to a terminal glycogenolysis from the liver. It is most probable that it is due to a final reaction on the part of the adrenal to dropping temperature and approaching death. Geiger (1925) has shown that when a rabbit is cooled sufficiently to make its temperature definitely subnormal, then it develops a considerable hyperglycæmia, and Cramer has shown that the exposure of animals to cold produces great activity and final exhaustion of the adrenal and thyroid. These facts were not correlated by their respective observers, but when we put the two

together, they make a unity of the falling temperature and rising blood-sugar observed in our rabbits.

However, in rabbits (*cf.* rabbit 1) in which death was produced only a day or two after the injection of the diphtheria toxin, there was no prelethal rise in blood-sugar, and the adrenal would seem to have been paralysed from the commencement by the large dose. Mikami (1925) has found some interesting results with the injection of *intravenous* doses of diphtheria toxin in rabbits which are supplementary to our results. With large doses, fatal within 24 hours, an immediate hyperglycæmia was produced, which reached its maximum in three hours and then gradually decreased. We have produced similar hyperglycæmias with intravenous injection of vaccines. The intoxication depletes the adrenaline store, the loss being maximal at the sixth hour. Double splanchnectomy prevents both the depletion and the hyperglycæmia, so that the effect in this condition is of central origin. With a dose which was not fatal for 24 hours or longer he found no hyperglycæmia, but a slowly-developing hypoglycæmia (as in rabbit 1 of our series), during which time the suprarenal adrenaline and the liver glycogen diminished greatly. Splanchnectomy did not prevent these latter diminutions. It would seem that these various blood-sugar conditions found after poisoning with diphtheria toxin can be explained by the effect of the various doses in causing either a primary paralysis, a stimulation followed by exhaustion, or with the smallest doses merely a stimulation of the adrenaline reaction to toxæmias.

A similar prelethal rise accompanying a fall in temperature occurs even more strikingly in rabbits dying of anaphylaxis induced by bacterial sensitization. Zeckwer and Goodell (1925) record extremely high and rapidly produced hyperglycæmia just before death—a rise in one rabbit from 0·3 to 1·1% in the 18 minutes preceding death. They did not investigate the glycogen content of the liver and muscles, but O'Neill and others (1925) supplied the evidence that intense glycogenolysis occurs in anaphylaxis by showing that a complete disappearance of glycogen from the livers of dogs takes place within 15 minutes of the onset of anaphylaxis.

Diminution or failure of insulin action.—It is clear from the tables that, in nearly every curve done after the injection of diphtheria toxin, the hypoglycæmic action of insulin was less than before the injection, and indeed on some days was entirely absent. This is most obvious when the febrile reaction wore off and when the chronic effect of the toxin was fully established. It will be seen that the effect of insulin varied from day to day, and it is noticeable that, when the fasting level was low, insulin action was least. This is not invariable, but occurred in most of the rabbits recorded in the tables and in others as well. How can this be explained by what we know of insulin action and by the over-activity of the thyroid-adrenaline apparatus which we think is established in these cases?

Insulin has probably two actions which must be considered in the explanation of the above phenomena. In the first place it has been definitely shown to form glycogen in the muscles and liver when abundance of glucose and insulin is present. It has, however, not been shown to do so with small doses and with little blood-sugar to work on, as in the conditions of the above experiments. Perhaps it may do so, but the quantitative change in the

amount of glycogen would be too small to be clearly demonstrated. We cannot be certain, therefore, that the failure of insulin to reduce blood-sugar on certain days is not due to the failure to form glycogen on these days, and any estimations of liver or muscle glycogen are not likely to help us, because the changes would probably be too small to estimate. A failure to form glycogen, however, is not a likely explanation because, if the failure were due to the toxic action on the liver and muscle cells preventing glycogen synthesis, it is difficult to see why these cells should fail one day and act on a subsequent day (compare rabbits 7 and 8).

The explanation seems to lie in the second suggested action of insulin (suggested by Lesser, Dale and others), that it checks the new production of sugar from protein and perhaps from fat, and, we would add, from preformed glycogen. When the fasting blood-sugar is high in our experiments—a time when new production of blood-sugar would appear to be rapid because the activities of the rabbits were too constant to introduce another element which might raise or lower blood-sugar—insulin lowers the blood-sugar to about normal by checking the new production of sugar. When the blood-sugar is low, less production of new sugar is going on for insulin to check, and the blood-sugar remains constant. We have already suggested an explanation of this variability of the fasting blood-sugars by the varying activity of the thyroid and adrenal from time to time under the effect of the diphtheria toxin.

But this still does not explain fully why insulin fails to lower the initially low blood-sugar, because if it has checked the new production of sugar, we would expect the blood-sugar to fall gradually as it is used in tissue metabolism. It is noticeable that what insulin will do in this condition is to lower the blood-sugar to a low normal level, but no lower, and perhaps this is the key to the explanation. We know that a certain level of insulin hypoglycæmia produces a reactive mobilization of new sugar either directly from the liver glycogen or more probably through the stimulation of adrenaline. If the adrenal is over-active, as would seem to be the case after certain degrees of diphtheria poisoning, it may be that the adrenal reaction is produced more easily and at a higher blood-sugar level and refuses to allow the blood-sugar to sink at all below normal. Certainly, when the prelethal rise was occurring in rabbit 7, insulin acted quite strongly, but in rabbit 3 (second day after second dose) insulin seemed to stimulate quite definitely a rise in blood-sugar, because this rise did not go on continuously, but the blood-sugar fell to normal next morning before the real and final prelethal rise began. Also in rabbit 8 (third day) insulin seemed to start a definite though small rise in blood-sugar.

If the insulin is the cause of these rises—not merely that they occurred in spite of it—then they could only be due to an increased mobilization of sugar through stimulation of the over-sensitive adrenal. We may note in this connection that heavy doses of insulin have been shown to cause suprarenal enlargement in dogs (Riddle, 1924). We advance this explanation very tentatively at present as the most likely one, and are at present trying to elucidate the problem further by the use of ergotamine at various stages. But these rabbits respond very differently to the same dose of toxin and often die unexpectedly or at inconvenient times, so that we may not succeed for some

considerable time. Whatever the actual details of the mechanism, the most probable explanation of the variations in insulin action from day to day would appear to lie in the varying activity of the adrenal and thyroid, and the power of insulin to restrain their activity differently on different days.

The relation of blood-sugar to body temperature.—Two curious abrupt rises in blood-sugar with an accompanying drop in temperature were observed in rabbits 9 and 14. When the first was noticed it was thought that there must be some error in the blood-sugar estimation, although we could not see how it crept in. When the second occurred, and it was realized that both were accompanied by a fall in body temperature, the phenomenon seemed definitely established. One of these observations was made on a normal rabbit and the other after the injection of diphtheria toxin. Other observations on the connection between body temperature and blood-sugar have been recorded.

Geiger (1925) found that an artificially produced fall of temperature in rabbits produced hyperglycæmia, and Cramer has found that cooling stimulates the adrenal thyroid apparatus to increased activity—facts which no doubt have some connection. Cramer looks upon the thyroid adrenal apparatus as the regulator of temperature, its increased activity being the cause of pyrexia and febrile reactions. It is easy to understand in these two rabbits that a drop in body temperature might stimulate the adrenal and raise the blood-sugar, but the cause of the fall in temperature remains quite a mystery, especially in the normal rabbit, and we can find no record of similar observations by other workers. The low temperature would seem to be primary to the hyperglycæmia.

Marks has recorded the opposite conditions in thyroid-fed rabbits—a marked hypoglycæmia associated with hyperpyrexia. This occurred in thyroid-fed glycogen-free rabbits. He gave them a small dose of glucose which produced a profound and fatal hypoglycæmia, presumably by stimulating a large production of insulin which the glycogen-free rabbit was unable to balance. It is quite likely that this hypoglycæmia produced an intense adrenalin reaction, which was unable to raise the blood-sugar in the glycogen-free rabbit, but did raise the temperature to a hyperpyrexial level by burning all the available glucose. At any rate, there is some definite connection between body temperature, adrenal activity and blood-sugar concentration which requires further study.

THE EFFECT OF INFECTIONS ON THE BLOOD-SUGARS OF NORMALS.

If the deterioration in carbohydrate metabolism in diabetics during infections is due to the antagonistic activity of an over-stimulated thyroid adrenal apparatus, we would expect the same mechanism to be at work in infections in normal individuals, and might hope to see a similar disturbance of carbohydrate metabolism, though to a less obvious degree, with the more stable carbohydrate metabolism and large reserve insulin production of normals. Such changes must be expected to be very slight, but they are present apparently in all cases of infection when they are carefully looked for. We have noticed—and many other observers have made similar records—that the blood-sugar curve in chronic infective conditions is usually slightly

abnormal, showing a slight hyperglycæmia and a delayed return to the normal fasting value, giving indeed the type of curve which is associated with Graves's disease. Labbé and Boulin (1925) have made a careful study of the blood-sugar curve after 50 gm. of glucose in many various infections—typhoid, pneumonia, erysipelas, etc.—and found in them all slightly elevated and prolonged curves, sometimes accompanied by glycosuria and a slightly but definitely raised fasting blood-sugar concentration. This was not dependent on the severity of the disease, and indeed the disturbance in the blood-sugar curve was greater in mild than in severe typhoid. They gave no explanation of the phenomena observed, which we would again explain by a stimulation of the thyroid adrenal apparatus which is constantly found in infections. Berg (1926) has investigated the blood-sugar in normal individuals suffering from tuberculosis. He finds a high normal fasting blood-sugar and definite hyperglycæmia and prolonged blood-sugar curve in many cases. Active cases, and especially those with exudates, are more abnormal than stationary ones. He states that a large percentage of those cases with definitely abnormal blood-sugars showed symptoms suggestive of hyperfunction of the thyroid. Dr. Fremont-Smith, of Harvard, has recently told one of us in a personal communication that an extensive investigation of the fasting blood-sugar level has been made in infections, and that it has been found to be slightly, but definitely and invariably, raised in all conditions in which pyrexia is present. These small deviations from the normal are usually missed in non-diabetic individuals unless they are carefully looked for. But in individuals who presumably already have some slight abnormality of carbohydrate tolerance and pancreatic power, the onset of a bacterial infection produces an apparently severe and temporary diabetic condition which clears up on removal of the sepsis, as so frequently happens in connection with carbuncles in the elderly and in mild diabetics.

INSULIN-RESISTANT CASES OF DIABETES.

Apart from cases of diabetes complicated with infections, a number of other cases have been recorded as being unusually resistant to insulin action and therapy. We have analysed as many of these cases as we could find with a view to finding any factors that would account for their failure to respond to insulin in the usual way, and, as the following cases will show, have found evidence of an over-active thyroid adrenal apparatus in more cases than not. Indeed, apart from two cases of diabetes reported by Adams (1925), which were complicated by pernicious anæmia, the only complicating factor that can be gathered from the reports of these insulin-resistant cases is this thyroid and (or) adrenal element. We do not suppose, however, that we have been able to trace all insulin-resistant cases that have been recorded.

Frank (1926) reports only two clear diabetic cases of this type and neither of them was absolutely resistant to insulin; but the hypoglycæmic action of insulin and its glucose equivalent in diet were far below normal. One was an excitable neuropath, and the other a woman who suffered from paroxysmal tachycardia, sweats and easily-induced excitement—both obviously of the type where the sympathetic nervous system is over-active, without there being any definite enlargement of the thyroid gland noted in the records.

Pollak (1925) states that diabetes associated with hyperthyroidism is very resistant to insulin, and that, after X-rays to the thyroid gland, these cases respond much better to insulin. He also states that cases of "hypo- or dysfunction" of the thyroid may also be abnormally resistant to insulin, and quotes a case of thyrogenic adiposity and diabetes in whom 100 units a day produced no effect. The significance of this is not clear to us.

Falta (1923) also states that cases of diabetes complicated with hyperthyroidism and vascular hypertonia mostly show a relative resistance to insulin, and Radoslav cites such a case in which 180 units a day made no difference to the daily excretion of 80 gm. of glucose on a constant diet.

Mahler and Pasterny (1924) report a case of diabetes which was strikingly resistant to insulin. This case was demonstrated in 1920 as diabetes complicated with pituitary tumour, causing blindness, and thyroid enlargement. The pituitary tumour recurred after an operation for its removal and the diabetes became very severe. The fasting blood-sugar varied from 290 to 400 mgm. per cent. and 40 to 60 units of insulin had no effect on it.

A case of Graves's disease already reported by one of us (Lawrence, 1924) showed the antagonistic effect of thyroid stimulation on insulin action in a very clear manner. This woman at first showed the disturbance of carbohydrate metabolism which is typical of Graves's disease—a hyperglycæmia and glycosuria after meals, but a normal fasting blood-sugar. She was treated with 100 units of insulin a day—not for diabetes, but to see the effect on Graves's disease—and on this became aglycosuric and had normal blood-sugars all day. She was extremely upset one day by bad news from home and developed an acute exacerbation of her hyperthyroid condition (increased size and pulsation in the gland, increased pulse-rate, nervousness, etc.). At the same time, without any change in the diet or insulin, a heavy glycosuria appeared and definitely diabetic blood-sugars—179 mgm. per cent. fasting and 372 as the highest figure recorded after a meal. The carbohydrate of the diet was reduced from 450 to 200 gm. per day and the insulin kept the same. In spite of this it was not until the emotional crisis disappeared that her blood-sugars returned to normal levels. Soon after this an attempt was made to withdraw the insulin, but she had become definitely diabetic by this time, returned home refusing to use insulin and died in coma a few months later. It was very striking the way in which, in a day or two, her thyroid crisis induced a severe diabetes on what had appeared before to be merely the postprandial temporary hyperglycæmia of Graves's disease.

Within the last three weeks another case has occurred which shows still more clearly the effect of thyroid activity on insulin action. A woman of 30 was recently admitted to King's College Hospital in diabetic coma with the following history. In 1922 she was treated medically in Guy's Hospital for Graves's disease. In January, 1924, one of us (R. D. L.) commenced her insulin treatment for severe diabetes—apparently a sequel to the Graves's disease. Up to September, 1926, she kept well and active on about 40 to 60 units of insulin a day. She often had sugar in the urine when she failed to keep her diet and was a diabetic of extreme severity, although she could dance and play tennis. About September 10th, 1926, her thyroid began to enlarge, she lost her appetite, had severe headaches and greatly increased exophthalmos.

By September 19th she had developed air-hunger and persistent vomiting in spite of continuing 50 units a day. She was admitted to hospital almost unconscious with severe ketosis. No infection could be found, but the thyroid was considerably enlarged, the eye-signs were all present and the pulse was 120 to 130 per minute. She soon recovered from her comatose condition with insulin and rectal glucose. Seven days later she was requiring 130 units a day (in four doses) on a diet containing 45 gm. of carbohydrate, which, in the past, 40 units a day had been sufficient to control. Her pulse remained at 110 and other symptoms of Graves's disease persisted. Lugol's iodine, 10 minims *t.d.s.*, was commenced, and three days later the pulse had begun to fall (99/89) and 80 units a day caused hypoglycæmic symptoms. The hyperthyroid symptoms continued to disappear, and by the seventh day of iodine treatment her pulse was 70/75, and 60 units of insulin in three doses brought her blood-sugar down to 0·120 per cent. at noon and 0·084 per cent. at 4 p.m. A week previous the lowest observed blood-sugar with 130 units of insulin was 0·150 per cent. This improvement in seven days in insulin action from a requirement of 130 to 60 units was directly due to the control of her hyperthyroid condition by iodine.

Another interesting insulin-resistant case without any obvious infection is reported recently by Minkowski (1926). This was a diabetic on a fixed diet whose condition was satisfactorily controlled by insulin. She became resistant to insulin, and at the same time it was noticed that her blood-pressure was rising and Loewi's adrenalin eye-reaction became strongly positive—both evidence of increased sympathetic tonus, probably due ultimately to increased adrenalin secretion. It was found that ergotamine greatly increased the insulin action in this case, presumably by controlling the increased adrenaline output, or at least the increased sympathetic tonus.

Most of these cases show clearly, and all of them suggest, that, apart from infective conditions, the failure of insulin action in diabetes is found in cases with increased sympathetic action, occurring most clearly in obvious hyperthyroidism, probably in hyperadrenalism (which is, however, a syndrome at which we can only guess), and in one case of pituitary disturbance. The well-known effect of worry in causing glycosuria, and even diabetes, is interesting in this connection. "When shares go down in Wall Street, diabetes goes up" is an apothegm which has point as well as poignancy. It would appear that glycogenolysis, or the direct new production of sugar, is so active that insulin is ineffective in its usual hypoglycæmic action. And we would suggest that in infections also it is the stimulation of the thyroid adrenal apparatus, for which we have brought forward so much evidence, that inhibits or antagonizes insulin action.

Whether there is a form of non-pancreatic diabetes, as has been suggested by many different workers, and whether it is these non-pancreatic cases which fail to respond to insulin, is not at all clear. It is obvious that the influences of the thyroid adrenal apparatus which we have described have a tendency to produce conditions resembling mild diabetes and to develop latent diabetes. But we should imagine that these influences, acting on a normal pancreas, would never produce diabetes unless they act long enough to wear out the pancreas, as fairly frequently happens in Graves's disease or acromegaly,

where a permanent and true diabetes may supervene. The possibility of these influences producing blood-sugar curves which resemble mild diabetes should always be kept in mind, and prevent us placing too much reliance in slight deviations from the normal blood-sugar curve in the diagnosis of diabetes.

CONCLUSIONS.

It would appear that there is a mechanism in the thyroid and suprarenal glands—the "thyroid-adrenal apparatus"—which, when stimulated, is a direct physiological antagonist to insulin action. This mechanism is stimulated by toxæmias, and particularly by bacterial infections, and is an adequate explanation of the increase of severity in diabetes, and the failure of insulin action, either relative or absolute, in these conditions. There is no need to postulate that infections lower the amount of insulin produced by the pancreas or that the toxins destroy insulin by direct chemical action. The mechanism is also at work in normal individuals, but less clearly, as is seen from the slight rise in the fasting blood-sugar concentration and the definitely abnormal blood-sugar curves during infections of many different types. Over-activity of the thyroid adrenal apparatus also accounts for the disturbance of carbohydrate metabolism in hyperthyroidism and probably in hyperpituitarism. It accounts for the raised blood-sugar curves and high blood-sugars found in conditions of excitement, and explains the effect of worry and nervous strain as a factor in causing and increasing the severity of diabetes. It probably accounts for the prolonged deterioration in carbohydrate metabolism that is frequently observed after shock, operations and such anæsthetics as ether, which we have noticed especially in nervous diabetics.

We would suggest the following as the detailed mode of action of these two opposing factors regulating carbohydrate metabolism. On the one hand, insulin, produced by the stimulus of ingested carbohydrate and a raised blood-sugar, stores away this sugar as glycogen in the liver and muscles, and at the same time probably checks the new formation of sugar from protein and perhaps fat. On the other hand, the thyroid adrenal apparatus, stimulated by toxæmias or a number of other factors, mobilizes sugar from stored glycogen and hastens the production of new sugar from other substances. We know that adrenaline does both these things directly, but are not certain whether the thyroid acts directly or through the stimulation of the adrenals. It is probable that the adrenal is the immediate mode of reaction and thyroid the prolonged reaction to stimuli which excite the combined mechanism. We see that insulin and the thyroid-adrenal apparatus are therefore direct antagonists in their effect on carbohydrate metabolism, the first being anabolic, the second catabolic. In cases of diabetes complicated by infections, it depends on the degree of reaction stimulated by the type and severity of the infection whether insulin can lower the blood-sugar at all or is entirely antagonized in this respect by the opposing mechanism.

PRACTICAL AND THERAPEUTIC APPLICATIONS.

From the theory that insulin action is opposed by an over-stimulated thyroid adrenal apparatus in toxæmias, two practical indications follow quite logically, and depend on the removal or depression of the stimulating toxæmia.

In the first place, the most satisfactory way is to remove or reduce the infection by all the therapeutic measures at our command. This is a well-established practice, and consists in immediate removal of septic foci and collections of pus as far as lies within our surgical power. The draining of an abscess or opening of a quinsy has an immediate and powerful effect in increasing insulin action. One can count implicitly on being able to reduce the dose of insulin as soon as a toxic focus is removed, and, indeed, must do so in order to prevent the onset of hypoglycæmia that the continuation of large doses of insulin would certainly induce.

In the second place, when the toxæmia is widespread or therapeutically irremovable, the only indication is to reduce its stimulating effect as far as possible. Apart from the use of iodine or X-rays in directly controlling an obvious complicating hyperthyroidism, as in the case already described, the only drug which we know to have any controlling action on an over-active thyroid-adrenal apparatus is ergotamine. In the few insulin-resistant cases in which we have so far used it, it has definitely increased insulin action in lowering blood-sugar, but far wider experience will be necessary to decide if it has any definite therapeutic value in this respect. From what we know of the action of ergotamine in rabbits, very large doses are necessary to depress sympathetic action to any great extent and entirely to inhibit all glycogenolysis. In insulin-resistant diabetics we have been using the preparation femergin hypodermically in doses of only 2 c.c. at a time (1 c.c. contains 1 mgm. of ergotamine tartrate). Even in this dose it has caused slight headache and a feeling of nausea, and it seemed inadvisable to push the drug further. Moreover, if we judge by its slowing of the pulse-rate, the effect of femergin does not last long, being maximal in about half an hour and passing off entirely in three to six hours. Now the doses which are necessary in rabbits to produce a really powerful paralysis of liver glycogenolysis are about 30 times—weight for weight—the amount we have felt justified in giving to human beings therapeutically. It therefore seems likely that with the clinical doses we have so far used only a very slight inhibition of glycogenolysis and new sugar production could be obtained, the effect of which it is difficult to demonstrate clearly, and still more difficult to assess in its therapeutic value or possibilities. We have already obtained encouraging results in every one of the insulin-resistant cases which we have tried, but such cases are infrequent. It is probable, too, that ergotamine will increase insulin action in every case of diabetes in varying degrees, as it does experimentally in animals. It must therefore be a matter of prolonged investigation to determine whether its action is depressing the thyroid adrenal apparatus can be used to much clinical advantage. We incline to think not.

SUMMARY.

1. A condition has been produced in rabbits with diphtheria toxin in which (*a*) insulin action is reduced or absent, (*b*) a prelethal rise in blood-sugar occurs, which has been reduced by ergotamine (femergin) and is therefore due to glycogenolysis, (*c*) and in which striking histological changes have been found in the thyroid and adrenal.

2. The effect of toxæmias and infections on the thyroid and adrenal ("the

thyroid-adrenal apparatus") is discussed, and it appears that these glands are always stimulated by toxæmias. It is suggested, and seems clear, that the overactivity of these two glands, both physiological antagonists to insulin, is the cause of the failure of insulin action during infections in diabetics. The effect of infections on the blood-sugar of non-diabetics is also discussed.

3. A number of insulin-resistant cases of diabetes, other than infected ones, are analysed, and most are clearly found to be complicated by an overactive thyroid or adrenal gland.

4. This theory of the overactivity and antagonism of the thyroid and adrenal during infections to insulin action is briefly discussed in its therapeutic aspects.

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SIMULTANEOUS DETERMINATIONS OF ARTERIAL AND VENOUS BLOOD-SUGARS IN DIABETIC INDIVIDUALS.*

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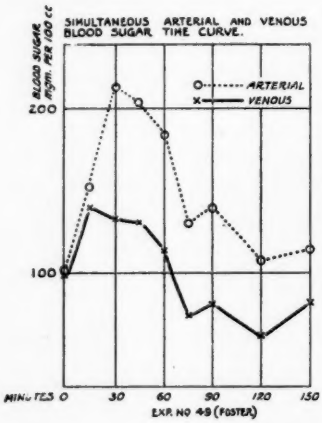
FOSTER (1923) has shown that in normal individuals, following glucose ingestion, the concentration of sugar in arterial blood rises to a higher level than in venous blood. Arterial and venous blood-sugar time curves, obtained simultaneously, present the following characteristics: In the fasting state the sugar contents of both bloods are the same. Shortly after ingestion of glucose the curves diverge. The greatest divergence is noted within the first hour. The curves then approach each other, but that of the venous blood-sugar reaches the fasting level sooner than that of the arterial. At the end of the third hour both show blood-sugar values below that of the fasting period, and here also the venous blood-sugar is lower than that of the arterial specimen. At times the arterial blood-sugar may fail to fall below the fasting level. One of these curves is reproduced in the accompanying chart.



As a substitute for pure arterial blood "finger blood" was employed. With the technique described for obtaining the latter, Foster has shown that, with regard to their sugar contents, "finger blood" and pure arterial blood are practically the same.

Prompted by these observations, Lawrence (1924) estimated, simultaneously, arterial and venous blood-sugars in diabetics to see if the above-mentioned normal differences were present. Nine experiments on three subjects—all severe diabetics—are recorded. Hyperglycæmia was found in both venous and arterial blood. The differences between the sugar contents of arterial and venous blood were, however, much less marked than in non-diabetics. With fasting blood-sugars, ranging between 0·14 and 0·17 per cent., little or no differences were noted, though with such concentrations, following glucose ingestion in normal individuals, 30 to 40 mgm. differences between arterial and venous blood could be recognized. Venous blood-sugars higher than arterial were noted in two instances. It would appear, according to Lawrence, that there are times when the tissues are discharging more sugar into the

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venous blood than they are receiving from the arteries. In a few mild diabetics, the data of which are not recorded, Lawrence found the differences between arterial and venous blood to be less than the normal, but greater than with severe diabetics. Administration of insulin increased the "difference values" to a degree approaching the normal. Because of these observations Lawrence suggested the possible value of simultaneous studies of arterial and venous blood-sugars in the diagnosis, prognosis and differentiation of various types of diabetes and hyperglycæmia.

Results obtained in experimental work on animals and of a somewhat similar nature were more recently recorded by Frank, Nothmann and Wagner (1925). These authors have shown that following intra-arterial injection of insulin in normal dogs there may be as much as 46 mgm. difference between the blood-sugar of the left ventricle and the femoral vein. Following the same experiment on depancreatized dogs the same phenomenon may be observed, although before insulin injection the blood-sugar in the left ventricle of the heart may be lower than that of the femoral vein.

The recognition of this phenomenon recorded by Foster opens up new fields for research, particularly in diabetes, and its importance may be seen from different angles. The fact that arterial and venous blood-sugars do not parallel each other following glucose ingestion may explain some of the discrepancies between the results of different workers in studying the renal threshold for sugar. It may also explain the fact frequently noted that many individuals, though not typical renal glycosurics, have glycosuria following ingestion of glucose with no *apparent* hyperglycæmia. These arterio-venous differences are also of interest in view of recent work on the different forms of glucose in the blood, particularly the influence of these different sugars on the rate of glycolysis. In a series of experiments (unpublished data) we have found that the rates of glycolysis of diabetic bloods were less in venous than arterial specimens. The immediate practical application of arterial and venous blood-sugar studies appears to be in the diagnosis and differentiation of the various types of diabetes, and the object of this report is to record results obtained along these lines.

METHOD OF INVESTIGATION.

Blood-sugar determinations of arterial and venous specimens were made upon thirty patients with glycosuria, in the fasting state firstly, and again thirty minutes after ingestion of 25 gm. of glucose. Since at the time of the investigation all subjects were regarded as diabetics, the reason for the use of such small quantities of glucose is obvious. It will, however, be recalled that, unlike the rate of recovery, the position of the peak of the blood-sugar time curve following glucose ingestion is not a function of the amount of glucose administered. The selection of the thirty-minute period was based upon the well-known fact that the maximum venous blood-sugar concentration is usually reached within that time in normal individuals. This also appears to apply to arterial blood. In all of Foster's experiments, with one exception (Exp. No. 44), the peaks were noted within one hour following glucose ingestion.

Pure arterial blood obtained from the radial artery was employed in this

study. The method described by Stadie (1919) for successful radial puncture has been employed in this laboratory for some time. The theoretical complications—thrombosis, embolism, aneurysm and hæmorrhage—have not been met with. Very slight hæmatomata have occasionally been noted. With the use of a hypodermic needle this difficulty is practically overcome. No local anæsthetics were used.

The combined data of the first thirty observations, tabulated in the order in which they were obtained, are recorded in Table I. In each case are recorded, in order, the arterial and venous blood-sugars, and the "arterio-venous difference values" in the fasting state and thirty minutes after glucose ingestion.

TABLE I.—*Arterial and Venous Blood-Sugar Percentages Before and After Ingestion of Glucose.*

Subject.	Fasting state.			30 min. after glucose.		
	Arterial.	Venous.	Difference.	Arterial.	Venous.	Difference
R. L—	0·120	0·116	0·004	0·256	0·227	0·029
J. B—	0·100	0·098	0·002	0·212	0·180	0·032
F. N. M—	0·228	0·221	0·007	0·315	0·285	0·030
M. K. K—	0·219	0·218	0·001	0·306	0·299	0·007
S. K—	0·169	0·171	0·002	0·342	0·338	0·004
S. C—	0·082	0·083	0	0·188	0·154	0·034
R. S. H—	0·290	0·297*	0·007	0·402	0·399	0·003
W. T. L—	0·122	0·120	0·002	0·288	0·256	0·032
E. McE—	0·210	0·218*	0·008	0·360	0·378	0·018*
R. R—	0·187	0·178	0·009	0·301	0·282	0·019
E. J—	0·100	0·097	0·003	0·251	0·192	0·059
F. W. H—	0·111	0·108	0·003	0·262	0·210	0·052
J. K—	0·162	0·166*	0·004	0·328	0·326	0·002
A. W. M—	0·099	0·088	0·011	0·244	0·212	0·032
F. P—	0·192	0·182	0·010	0·262	0·243	0·019
W. R—	0·215	0·226*	0·011	0·318	0·318	0
W. J—	0·112	0·110	0·002	0·288	0·256	0·032
I. R—	0·072	0·067	0·005	0·218	0·149	0·069
A. J. D—	0·153	0·148	0·005	0·299	0·288	0·011
L. K—	0·222	0·221	0·001	0·343	0·351	0·008*
J. P—	0·104	0·099	0·005	0·232	0·214	0·018
K. G—	0·082	0·078	0·004	0·220	0·162	0·058
W. L. P—	0·197	0·186	0·011	0·322	0·301	0·021
H. S—	0·133	0·132	0·001	0·336	0·332	0·004
M. L—	0·120	0·111	0·009	0·246	0·221	0·025
H. K—	0·149	0·144	0·005	0·264	0·252	0·012
H. McG—	0·121	0·120	0·001	0·258	0·178	0·080
R. W. R—	0·213	0·200	0·013	0·314	0·292	0·022
C. P. C—	0·126	0·126	0	0·226	0·214	0·012
I. L. L—	0·092	0·088	0·004	0·212	0·164	0·048

* Venous blood-sugar greater than arterial.

DISCUSSION OF RESULTS.

The fact that the concentration of sugar may be higher in arterial than in venous blood is obvious. In the fasting state differences between arterial and venous values may be entirely absent, and, if present, are much less marked than after glucose ingestion. In a few instances the venous values are higher than the arterial. Following glucose ingestion there is no uniformity in the results. In the great majority of cases the values of the arterial bloods are higher than those of the venous specimen. In one instance (W. R—) there was no difference, and in two cases (E. McE— and L. K—) the venous values were higher than the arterial. Without further analysis such data appear to yield little useful information. When, however, they are correlated with the clinical and laboratory data their usefulness is very suggestive.

A number of these individuals came for observation because of sugar having been found in the urine during the course of examination for life assurance. None of these subjects had clinical signs or symptoms suggestive of diabetes. In six cases the blood-sugar time curves following glucose ingestion were of the normal type. These six subjects may, therefore, be reasonably regarded as non-diabetic, and will be considered as a group by themselves. The arterial and venous blood-sugars and the "arterio-venous" differences in the fasting state and thirty minutes after glucose are recorded in Table II. It will be noted that in the fasting state the blood-sugars were

TABLE II.—*Normal Individuals. Blood-Sugar Per Cent.*

Subject.	Fasting state.			30 min. after glucose.		
	Arterial.	Venous.	Difference.	Arterial.	Venous.	Difference.
J. B—	0·100	0·098	0·002	0·212	0·180	0·032
S. C—	0·082	0·083	0	0·188	0·154	0·034
I. R—	0·072	0·067	0·005	0·218	0·149	0·069
K. G—	0·082	0·078	0·004	0·220	0·162	0·058
H. McG—	0·121	0·121	0·001	0·258	0·178	0·080
I. L. L—	0·092	0·088	0·004	0·212	0·164	0·048
				Average		0·053

TABLE III.—*Diabetics, Groups* 1 *and* 2. *Per Cent. Blood-Sugar.*

Subject.	Fasting period.			30 min. after glucose.		
	Arterial.	Venous.	Difference.	Arterial.	Venous.	Difference.
C. P. C—	0·126	0·126	0	0·226	0·214	0·012
E. J—	0·100	0·097	0·003	0·251	0·192	0·059
M. L—	0·120	0·111	0·009	0·246	0·221	0·025
F. W. H—	0·111	0·108	0·003	0·262	0·210	0·052
W. T. L—	0·122	0·120	0·002	0·288	0·256	0·032
W. J—	0·112	0·110	0·002	0·236	0·217	0·019
A. W. M—	0·099	0·088	0·011	0·244	0·212	0·032
J. P—	0·104	0·099	0·005	0·232	0·214	0·018
R. L—	0·120	0·116	0·004	0·256	0·227	0·029
				Average		0·031

TABLE IV.—*Diabetics, Group* 3. *Per Cent. Blood-Sugar.*

Subject.	Fasting period.			30 min. after glucose.		
	Arterial.	Venous.	Difference.	Arterial.	Venous.	Difference.
F. P—	0·192	0·182	0·010	0·262	0·243	0·019
R. W. R—	0·213	0·200	0·013	0·314	0·292	0·022
F. N. M—	0·228	0·221	0·007	0·315	0·285	0·030
W. L. P—	0·197	0·186	0·009	0·322	0·301	0·021
R. R—	0·187	0·178	0·009	0·301	0·282	0·019
				Average		0·022

TABLE V.—*Diabetics, Group* 4. *Per Cent. Blood-Sugar.*

Subject.	Fasting period.			30 min. after glucose.		
	Arterial.	Venous.	Difference.	Arterial.	Venous.	Difference.
H. S—	0·133	0·132	0·001	0·336	0·332	0·004
J. K—	0·162	0·166	—0·004	0·328	0·326	0·002
H. K—	0·149	0·144	0·005	0·264	0·252	0·012
A. J. D—	0·153	0·148	0·005	0·299	0·288	0·011
S. K—	0·169	0·171	—0·002	0·342	0·338	0·004
				Average		0·007

TABLE VI.—*Diabetics, Group* 5. *Per Cent. Blood-Sugar.*

Subject.	Fasting period.			30 min. after glucose.		
	Arterial.	Venous.	Difference.	Arterial.	Venous.	Difference.
L. K—	0·222	0·221	0·001	0·343	0·351	—0·008
E. McE—	0·210	0·218	—0·008	0·360	0·378	—0·018
W. R—	0·215	0·226	—0·011	0·318	0·318	0
R. S. H—	0·290	0·297	—0·007	0·402	0·399	0·003
M. K. K—	0·219	0·218	0·001	0·306	0·299	0·007

normal, and the differences between arterial and venous values were hardly appreciable. After glucose ingestion marked differences were noted, in one instance amounting to 80 mgm. sugar per 100 c.c. blood (H. McG—). The average value of all the differences was 53 mgm. This is more than twice that of the remaining or diabetic group, which, excluding the two cases showing higher venous than arterial values, was approximately 20 mgm. When the diabetics are placed in separate groups according to the severity of the disease, there appears to be a definite relation between the magnitude of the "arterio-venous" differences and severity.

The classification used in this investigation was the one which has been employed in this clinic for a number of years, and on the whole has proven dependable. It was based upon findings with reference to glycosuria, acetonuria, clinical signs and symptoms, and the influence of diet. In this classification are excluded the acute and juvenile forms of the disease, and the mild diabetic whose tolerance may suddenly be lowered by trauma or infection. It is as follows:

Group 1.—The glycosuria is transient and may not be present for intervals of days. Acetonuria is absent. There are no other clinical signs and symptoms suggestive of diabetes. The sugar in the urine is usually discovered accidentally. The urines of these individuals are readily rendered sugar-free by avoiding excessive intake of carbohydrates and lowering the total caloric content of the diet. This is done by eliminating pastries, pudding, candies, avoiding sugar in tea and coffee, and taking no more than one slice of bread at each meal. By this procedure the average intake of carbohydrates is about 150 gm. a day, and these individuals usually have a tolerance for this amount at least.

Group 2.—The glycosuria is post-prandial. It is found regularly, two or three hours after a meal. The morning urines are sugar-free, and the fasting blood-sugars are normal. There is no acetonuria. There are no clinical signs or symptoms of diabetes other than the glycosuria. The glycosuria in these cases is also, as a rule, discovered accidentally. These individuals are able to tolerate between 100 and 150 gm. of carbohydrates a day.

Group 3.—The glycosuria is persistent. It is found throughout the day and also in the early morning specimen. There is hyperglycæmia in the fasting state. These individuals still have a fair tolerance for sugar and therefore have no acetone bodies in the urine. They may or may not have clinical signs and symptoms of the disease. Following one or two days' starvation or "green-day" diet, which in this hospital consists of 15 gm. of carbohydrates, 20 of fat and 24 of protein, the urines of these individuals usually become sugar-free. By gradually increasing the diet they are able to tolerate from 75 to 100 gm. of sugar and keep their urine sugar-free and fasting blood-sugars within the normal limits of variation.

Group 4.—The glycosuria is persistent throughout the day. Acetone bodies are present in the urine. The clinical signs and symptoms are more exaggerated. The urines of these individuals can still be kept sugar-free, their blood-sugars normal, and the average height-weight relationship can be maintained on diet *without the use of insulin.*

Group 5.—These present an exaggeration of the picture of Group 4. *They require insulin to meet their daily requirements.*

Group 6.—These patients are in a pre-comatose state.

Group 7.—Coma.

All the subjects of this investigation were grouped according to the above classification, and are so recorded in Tables III to VI. Groups 1 and 2, on account of the uncertainty of the history of the frequency of glycosuria, are combined in one table.

It will be noted that the average values of the arterio-venous differences *following glucose ingestion* decrease as Groups 1 to 4 are observed in order, being 31, 22, 7 mgm., etc. Group 5 consists of the most severe of the diabetics observed. It will be noted that there is no difference between the arterial and venous blood in one case, and in two instances the venous bloods were actually higher than the arterial.

It appears a reasonable assumption, *a priori*, that the magnitude of the difference between the arterial and venous blood-sugars, following glucose ingestion, is a function of the severity of the disease. It is generally regarded

that glucose is supplied to the tissues by the arterial blood. If either oxidation or storage is taking place in the tissues, it is obvious that the (venous) blood leaving the tissues will contain less glucose than the (arterial) blood entering them. Since the rate of utilization of glucose parallels the severity of the disease, the greater the rate the greater will be the difference between the arterial and venous blood-sugar contents. (By utilization is here meant the combined mechanism of oxidation and storage.)

There appears to be no perfect correlation between the fasting venous blood-sugars and "arterio-venous difference values" following glucose ingestion. It will be noted that the average value of the former in Group 3 is higher than that of Group 4, whereas the average value of the latter is also higher in Group 3 than in Group 4. This is further proof, if any is necessary, that the degree of hyperglycæmia in the fasting state is not necessarily an indication of the severity of the disease. Mild diabetics are known to take enormous quantities of food, have marked fasting hyperglycæmias, excrete 200 to 300 gm. of sugar in a day, and become sugar-free after two or three days' starvation.

Further analysis of the arterio-venous differences in the fasting state shows some interesting results. It will be observed in Group 3, in spite of the marked fasting hyperglycæmias, there are some differences between the arterial and venous values, whereas with the fasting hyperglycæmias in Group 4, not only are the arterial bloods not higher than the venous in two instances, but one of the venous values (J. K—) appears to be higher than the fasting arterial, suggesting that the tissues in this case are adding more sugar to the venous blood than is being supplied to them by the arterial. The fact that in the subjects of Group 3 the arterial bloods are still higher than the venous, in spite of the hyperglycæmias, suggests utilization of glucose by the tissues, and therefore appears again to be an indication, as stated before, that there is no perfect correlation between the degree of hyperglycæmia and the severity of the disease.

Venous values greater than the arterial were again observed in the most advanced stage of the disease, namely, coma. In the following table are shown the arterial and venous blood-sugar values in three such cases:

Arterial and Venous Blood-Sugar in Diabetic Coma.

Number.	Arterial.	Venous.
1	0·540%	0·552%
2	0·400%	0·412%
3	0·588%	0·617%

It appeared from the above observations that simply estimating the arterial and venous blood-sugars in the fasting state would be sufficient to estimate severity, and to determine whether an individual does or does not require insulin. To test this a large series of diabetics was examined in the fasting state. The selection was random, the subjects—regular attendants and new patients—having been examined in the order in which they came to the diabetic out-door clinic. In Table VII are shown the results of the first fifty cases.

TABLE VII.—*Arterial and Venous Blood-Sugars (Per Cent.) During Fasting State.*

O.D. number.	Blood-sugar per cent.		Remarks.	O.D. number.	Blood-sugar per cent.		Remarks.
	Arterial.	Venous.			Arterial.	Venous.	
1725–23	0·184	0·182	—	21322–24	0·213	0·211	Requires insulin.
4312–26	0·211	0·212	—				
4171–23	0·230	0·232	Requires insulin.	1984–25	0·200	0·200	—
				6297–24	0·180	0·164	—
11736–20	0·172	0·169	—	6821–22	0·184	0·178	—
13839–20	0·313	0·312	—	6777–19	0·194	0·192	—
7202–26	0·215	0·216	—	5661–26	0·176	0·176	—
5433–26	0·193	0·189	—	946–23	0·199	0·184	—
20530–24	0·186	0·178	—	3903–25	0·211	0·210	—
7864–25	0·167	0·164	—	15670–24	0·202	0·200	—
A. S. J—	0·232	0·231	Requires insulin.	6437–25	0·333	0·326	—
				10282–15	0·302	0·301	—
13825–20	0·243	0·242	—	A. S. R—	0·219	0·217	—
M. L. P—	0·282	0·276	—	919–26	0·170	0·169	—
11662–14	0·253	0·254	—	16473–21	0·302	0·303	—
5193–26	0·298	0·310	Requires insulin.	12444–22	0·187	0·185	—
				5629–23	0·229	0·226	Requires insulin.
11955–24	0·170	0·164	—				
8978–23	0·156	0·154	—	A. McG—	0·283	0·281	—
19346–24	0·144	0·139	—	E. R—	0·154	0·154	—
6124–26	0·179	0·178	—	10499–24	0·199	0·200	—
6288–23	0·156	0·156	—	6594–23	0·213	0·201	—
5575–23	0·182	0·179	—	15392–22	0·180	0·169	—
626–23	0·221	0·222	Requires insulin.	3841–23	0·186	0·185	—
				7004–26	0·216	0·217	—
953–25	0·212	0·201	—	13365–24	0·188	0·175	—
14813–23	0·222	0·216	—	A. K—	0·146	0·146	—
7841–19	0·197	0·198	—	1079–24	0·232	0·229	—

It will be noted that of the fifty subjects, six required insulin. In all of these six cases with one exception (5193–26) the arterial and venous blood-sugars were practically alike. In the one exception the venous blood value was higher than the arterial. Of the remaining forty-four cases, practically no arterio-venous differences were found in thirty-one instances. (Differences of 1 to 3 mgm. were neglected for technical reasons.) In the remaining thirteen cases differences of from 4 to 16 mgm. were found. Since all of the forty-four patients are known to be able to do without insulin, the finding of identical arterial and venous blood-sugar values in the fasting state is of limited value for this purpose.

It would appear that in the fasting state the hyperglycæmia is due to a form of glucose not readily utilized, and the small amount of sugar which is being oxidized is liberated into the arterial blood at a rate equal to the rate of oxidation. The result of this is an absence of appreciable differences between

arterial and venous blood values. It would appear that only after absorption of comparatively large amounts of glucose, the ability to store or oxidize is reflected in the arterio-venous differences.

The points of practical interest here are the following :

(*a*) The finding of a venous blood-sugar value higher than arterial appears to be reliable evidence of severe diabetes.

(*b*) Either such finding in the fasting state or absence of any arterio-venous differences in the presence of hyperglycæmia *following glucose ingestion* appears to be an indication for the use of insulin.

It is obvious, however, that more data must be accumulated before these conclusions are proven to be wholly correct.

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PROTEINS OF THE CEREBRO-SPINAL FLUID.

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LITTLE is known concerning the quantitative aspect of the proteins in the cerebro-spinal fluid, although many theories are current.

Lange (1912) assumed that in luetic and paretic affections specific proteins occur in the cerebro-spinal fluid, and these abnormal proteins cause the well-known colloidal gold precipitation. Matzkiewitsch (1914) considered that peptone and not protein was formed in the fluid in certain syphilitic conditions, since the unusual constituent passed through the dialysing membranes he used. Felton (1917), in a short note containing no experimental evidence, concludes that abnormal relative amounts of globulin and albumin are present in the cerebro-spinal fluid which give a colloidal gold reaction. That globulin occurs in luetic and paretic fluids in abnormally large amounts is also the conclusion of Weston (1916). Cruickshank (1920) and Mellanby and Davies (1923) come to conclusions similar to those of Felton and Weston. It is to be noted that the work mentioned is not based on

accurate quantitative analyses. Ayer and Foster (1921) have determined by a nephelometric method the total protein content of the fluid in a large number of cases, but no attempt was made to determine the amounts of the different proteins present. The precipitin reaction has been applied to the study of the cerebro-spinal fluid by Hektoen and Neymann (1920, 1919–1923), but the results were not of a clear-cut quantitative nature. Mestrezat (1912) has attempted to determine the amount of albumin and globulin in the fluid in a number of cases, but his difficult and inaccurate method of combining evaporation and nephelometric measurements renders his results of limited value only. An accurate method of determining total proteins and the albumin and globulins in small quantities of cerebro-spinal fluid therefore was sought. Such a method was found by modifying the Wu method of analysis of plasma proteins. The application of Wu's method to the determination of the total protein content of the fluid has been described since this work was commenced (Ling, 1926), but no attempt was made by him to determine the separate protein fractions, and a small number only of fluids was examined.

The full experimental details are given later, but briefly the method consists in precipitating the globulins from the fluid by addition of the appropriate amount of ammonium sulphate and determination of the protein content of the original fluid and in the filtrates from the ammonium sulphate precipitation. This is done by precipitation of the proteins by tungstic acid, and measuring the colour produced by the precipitates with a phenol reagent. An accurate determination of the total protein-content alone may be performed on 2 c.c. of the fluid.

TABLE I.—*Total Protein and Albumin Content of Cerebro-Spinal Fluids.*

Case No.	Diagnosis.	Total protein. (%.)	Albumin. (%.)
1	Enteritis	0·033	0·029
2	Myelomatosis	0·040	0·037
3	Parenchymatous nephritis	0·031	—
4	?	0·040	—
5	?	0·035	—
6	Pleurisy	0·027	—
7	?	0·037	—
8	?	0·030	—
9	T.B. meningitis	0·226	0·182
10	,, ,,	0·093	0·075
11	,, ,,	0·133	0·108
12	,, ,,	0·093	0·068
13	,, ,,	0·170	—
14	Cerebral tumour	0·063	0·050
15	,, ,,	0·094	0·087
16	Encephalitis lethargica	0·110	0·083
17	,, ,,	0·046	0·034
18	Cerebral tumour	0·050	—
19	,, ,,	0·060	—

TABLE I—*continued.*

Case No.	Diagnosis.	Total protein. (%.)	Albumin. (%.)
20	Hydrocephalus	0·050	0·042
21	Tabes dorsalis	0·088	0·064
22	,, ,,	0·112	0·083
23	,, ,,	0·048	0·037
24	,, ,,	0·076	—
25	,, ,,	0·139	—
26	,, ,,	—	0·059
27	,, ,,	0·050	—
28	General paralysis	0·062	0·039
29	,, ,,	0·105	0·058
30	,, ,,	0·134	0·074
31	,, ,,	0·117	0·073
32	,, ,,	0·115	0·059
33	,, ,,	0·073	0·044
34	,, ,,	0·085	0·044
35	,, ,,	0·093	0·051
36	,, ,,	0·097	0·050
37	,, ,,	0·088	0·051
38	,, ,,	0·060	0·040
39	,, ,,	0·089	—
40	,, ,,	0·226	0·121
41	,, ,,	0·100	0·061
42	,, ,,	0·106	0·049
43	,, ,,	0·077	0·044
44	,, ,,	0·219	0·126
45	? Aortitis	0·050	0·040
46	? Myelitis	0·193	0·176
47	Uræmia	0·051	0·040
48	? Pyloric ulcer	0·073	0·062
49	Cerebral thrombosis	—	0·079
50	?	0·068	—
51	?	0·053	—
52	?	0·064	—
53	?	0·079	—
54	?	0·084	—
55	? Glioma sp. cord	0·054	—
56	?	0·056	0·049

Table I gives the result of total protein determinations in 54 cerebro-spinal fluids carried out by this method and determination of the albumin fraction in 37. It will be seen that the total proteins in cerebro-spinal fluids vary from about 0·03% in normal fluids up to a maximum of 0·23% in certain meningitic and paretic patients, and the albumin is never present in concentrations of less than about 0·02–0·03%, and may be as much as 0·18%. In every case (except one of paresis) there is less globulin than albumin in

the fluid—a fact which is not generally realized. The chief noteworthy points are the protein-content of the normal fluid, *i. e.* about 0·02-0·03% (*cf.* Ayer and Foster, 1921), the high figures in tubercular meningitis (0·09–0·23%, mean = 0·14%), in tabes (0·05–0·14%, mean = 0·09%), general paralysis (0·06–0·23%, mean = 0·11%), and in cerebral tumours (0·05–0·09, mean = 0·07%). An increase in the protein content of the fluid would seem to be demonstrated in the one case* of uræmia examined (0·05% instead of 0·03%). It will be seen that total protein increase alone is not the chief factor in paretic fluid, and the powerful effect in precipitating colloidal gold must be attributed to other factors, since meningitic and other fluids often have a higher total protein content.

The most important conclusions from this work have been reached by examining the albumin : globulin ratios in various diseases, which are summarized in Table II. The normal ratio is probably about 8 : 1, as shown in the first two or three cases in the table, there being about 0·003% of globulin normally. The average ratio of albumin to globulin in cases other than tabes and paresis is 6 : 1, in the three cases of tabes studied the average ratio is 3 : 1, and in the sixteen cases of general paralysis of the insane the ratio is 1·3 : 1.

As indicated previously, it has been suspected for some nine years that in paretic affections, etc., there is a relative increase in the globulin content of the cerebro-spinal fluid, and these remarkably clear-cut and definite results provide ample proof of the truth of the theory. The origin and cause of this relative increase of globulin, especially in general paresis, is not clear, but a defensive mechanism would seem to be indicated. It seems to us unlikely that a mere leakage phenomenon accounts for the presence of the increased proteins, since albumins with their smaller molecular size would probably diffuse more readily, and thus the albumin : globulin ratio would increase and not decrease.

It is noteworthy that the smallness of the albumin to globulin ratio roughly follows the strength of the Wassermann reaction and the extent of precipitation of colloidal gold in the Lange test; *e. g.* to take the two extreme values of the ratio in general paresis:

Case.	Ratio.	W.R.	Cells.	Lange.
35	0·9 : 1	+++++	++	5555555542
32	2·0 : 1	+	+	5555432100

Cases 13 and 14, both of the same family, suffering from encephalitis lethargica, gave some colloidal gold precipitation—Case 13: 3455554321; Case 14: 0112322100—as would be expected from the low albumin : globulin ratio. So that all the fluids giving a luetic or meningitic colloidal gold curve (meningitis, tabes and encephalitis lethargica) have albumin : globulin ratios between 2·7 and 4 (9 cases average is 3·3 : 1). And in the fluids not belonging to these classes or to the paretic type the average ratio (10 cases) is 7 : 1.

* Since this paper was submitted for publication a further case of uræmia has been studied. The protein content was 0·143%, confirming the increase noted.

In two cases of anterior poliomyelitis examined lately, increases of protein content (0·047% and 0·144%) were found, but the albumin : globulin ratio remained normal (7 : 1 and 8 : 1 respectively).

TABLE II.—*Albumin and Globulin of Cerebro-Spinal Fluid.*

Case No.	Albumin. (%.)	Globulin. (%.)	Albumin : Globulin.	Diagnosis.
1	0·029	0·004	7 : 1	Enteritis.
2	0·037	0·003	12 : 1	Myelomatosis.
3	0·045	0·006	8 : 1	Uræmia.
4	0·182	0·046	4 : 1	T.B. meningitis.
5	0·068	0·025	3 : 1	,, ,,
6	0·108	0·025	4 : 1	,, ,,
7	0·075	0·018	4 : 1	Meningitis.
8	0·176	0·017	10 : 1	? Myelitis.
9	0·040	0·010	4 : 1	? Aortitis.
10	0·062	0·011	6 : 1	Pyloric ulcer.
11	0·042	0·012	4 : 1	Hydrocephalus.
12	0·050	0·013	4 : 1	Cerebral tumour.
13	0·083	0·027	3 : 1	Encephalitis lethargica.
14	0·034	0·012	3 : 1	,, ,,
15	0·087	0·007	11 : 1	Cerebral tumour.
16	0·049	0·007	7 : 1	?
17	0·064	0·024	2·7 : 1	Tabes.
18	0·083	0·029	2·8 : 1	,,
19	0·037	0·011	3·4 : 1	,,
20	0·044	0·033	1·3 : 1	General paralysis.
21	0·126	0·093	1·3 : 1	,, ,,
22	0·058	0·047	1·2 : 1	,, ,,
23	0·074	0·060	1·2 : 1	,, ,,
24	0·038	0·024	1·5 : 1	,, ,,
25	0·059	0·056	1·1 : 1	,, ,,
26	0·073	0·044	1·7 : 1	,, ,,
27	0·044	0·029	1·5 : 1	,, ,,
28	0·044	0·041	1·1 : 1	,, ,,
29	0·051	0·042	1·2 : 1	,, ,,
30	0·050	0·047	1·1 : 1	,, ,,
31	0·051	0·037	1·4 : 1	,, ,,
32	0·040	0·020	2·0 : 1	,, ,,
33	0·121	0·105	1·2 : 1	,, ,,
34	0·061	0·039	1·6 : 1	,, ,,
35	0·049	0·057	0·9 : 1	,, ,,
36	0·136	0·130	1·1 : 1	,, ,,

It is common knowledge that routine turbidimetric determinations of the total protein or globulin content of cerebro-spinal fluids are not accurate, and a number of discrepancies are found when the routine examination and an accurate analysis are performed on the same fluid.

Determination of the pseudo- and euglobulin in the fluid separately is a matter of some difficulty. In normal people or people whose cerebro-spinal fluids show no abnormality the albumin content is probably about 0·02%,

the total globulin about 0·003% (a quantity which is only just detectable by this method), and the euglobulin about 0·0005%, which quantity cannot be analysed, especially since the figure is arrived at by difference. In fluids from meningitic, tabetic, etc., cases more globulins are present, and in general paralysis more still, so that analysis of the separate globulin fractions is more practicable. Table III shows the averaged results of such analyses conducted on 14 fluids, and the hypothetical analysis of a "normal" fluid estimated from a number of observations.

TABLE III.

Case.	No. of analyses.	Albumin. (%.)	Pseudo-globulin. (%.)	Euglobulin. (%.)
"Normal"	—	0·020	0·003	0·0005
Tabetic and meningitic	5	0·077	0·016	0·003
General paralysis	9	0·051	0·026	0·015

It will be noticed that in pathological fluids all the proteins are increased in quantity, but chiefly the globulins, and that in general paralysis, whilst the albumins had increased 2½ times and the pseudoglobulin 8 times, the euglobulin had increased about 30 times. In this connection, and as support for this typical analysis of a general paretic fluid, it may be mentioned that normal human plasma diluted 100 times in 0·9% sodium chloride solution gives the same colloidal gold curve in the Lange test as a paretic cerebro-spinal fluid, and the figures given in Table III for the general paretic fluid might well be that of 100 times diluted plasma. (1 c.c. plasma (diabetic patient) diluted to 100 c.c. with 0·9% NaCl solution, Wassermann reaction negative, gold curve (Lange) 5555542100.) Confirmation of our analyses is thus provided, and the view that colloidal gold precipitation is governed by albumin and globulin content is strengthened. It is hoped to deal at greater length with this aspect of the question in a further communication.

EXPERIMENTAL METHOD.

The modification of Wu's plasma protein analysis method is conducted as follows: 4 c.c. of fluid are sufficient to determine the total protein content and the albumin: globulin ratio of the cerebro-spinal fluid, but to carry out an analysis of the *three* protein fractions of the fluid at least 6 c.c. of clear fluid free from blood must be available. Three accurately measured samples are taken and placed in 25 c.c. centrifuge tubes. To the first tube, designated I, a few c.cs. of distilled water are added, then 1 c.c. of 10% sodium tungstate, and then, with stirring, 1 c.c. of 2/3 N. sulphuric acid. After standing for flocculation to commence, the tube is centrifuged, the supernatant liquid decanted off, and the precipitate dissolved in water to which 1 c.c. of sodium tungstate is added. Then 1 c.c. of 2/3 N. sulphuric acid is added and the precipitate collected as before.

To the fluid in tube II is added one half its volume of saturated ammonium sulphate solution to precipitate the euglobulin. The mixture is allowed to stand overnight, then centrifuged, the supernatant liquid poured off as completely as possible, its volume accurately measured, and the proteins precipitated twice with tungstic acid as previously described.

To the fluid in tube III is added an equal volume of saturated ammonium sulphate to precipitate the globulins. The clear supernatant fluid obtained by allowing to stand, centrifuging and decanting is measured and the proteins are twice precipitated with tungstic acid as before. The three purified tungstic acid-protein precipitates having been obtained, they are graded according to the amount of precipitate. Slight precipitates are made up to 10 c.c. volume in the subsequent colorimetric procedure and heavier precipitates are made up to 25 c.c. volume. Having graded the precipitates, they are each suspended in water, dissolved by addition of 1 drop of sodium carbonate, and transferred to stoppered measuring cylinders. 0·1 c.c. of Wu's phenol reagent is then added to the 10 c.c. cylinders and 0·25 c.c. to the 25 c.c. cylinders. 1 c.c. and 2·5 c.c. respectively of 20% sodium carbonate is added to each and the volumes made up to the prearranged mark (10 or 25 c.c.). At the same time standard tyrosine solutions are treated in the same way. The standard solutions used have been 0·2 c.c., 0·5 c.c. or 1·0 c.c. of 0·02% tyrosine dissolved in dilute hydrochloric acid and made up to 25 c.c., 0·25 and 2·5 c.c. respectively of phenol reagent and 20% sodium carbonate being added to each. After standing 20 minutes the protein solutions are read against the appropriate standard in a colorimeter in the usual manner.

CALCULATION OF RESULTS.

Data.—1 mg. of tyrosine gives the same coloration with the reagents as 27·5 mg. of albumin or 26·2 mg. of globulin (Wu, 1922).

Example.—The calculation is obvious but a little complicated, so an example will be taken.

Paretic Fluid.

Tube I.—Determination of albumin + pseudoglobulin + euglobulin:

2 c.c. of cerebro-spinal fluid. Proteins twice precipitated by addition of 1 c.c. each of 10% sodium tungstate and 2/3 N. sulphuric acid. Precipitate dissolved in dilute sodium carbonate solution in 10 c.c. measuring cylinder, 0·1 c.c. of phenol reagent and 1·0 c.c of 20% sodium carbonate solution added. Volume made up to 10 c.c. and colour compared with "1·0 tyrosine standard," *i.e.* 1·0 c.c. of 0·02% tyrosine solution with 0·25 c.c. of phenol reagent and 2·5 c.c. of 20% sodium carbonate solution; volume made up to 25 c.c.

25 mm. of tyrosine solution produced the same colour as 37 mm. of protein solution, *i.e.* protein solution corresponded to $\frac{25}{37} \times 1$ c.c. of 0·02% tyrosine solution, *i.e.* to $\frac{25}{37} \times 1 \times \frac{0{\cdot}02}{100}$ gm. of tyrosine; but since the volume of protein solution was 10 c.c. and that of the tyrosine solution 25 c.c., the protein is equivalent to $\frac{25}{37} \times 1 \times \frac{0{\cdot}02}{100} \times \frac{10}{25}$ gm. of tyrosine; further, 2 c.c. of the original fluid was taken, so that 100 c.c. would correspond to $\frac{25}{37} \times \frac{0{\cdot}02}{100} \times \frac{10}{25} \times \frac{100}{2}$ gm. tyrosine = 0·00270 gm. tyrosine = albumin, pseudo-globulin and euglobulin in 100 c.c. of fluid.

Tube II.—Determination of albumin + pseudo-globulin :

2·5 c.c. fluid and 1·25 c.c. saturated ammonium sulphate are added and allowed to stand overnight, then centrifuged and supernatant liquid poured off; 3·5 c.c. are taken and protein precipitated twice with tungstic acid as usual; final precipitate is dissolved in alkali in 10 c.c. measuring cylinder. 0·1 c.c. of phenol reagent and 1 c.c. of 20% $NaCO_3$ solution are added and compared with 0·5 standard tyrosine, *i. e.* 0·5 c.c. of 0·02% tyrosine, 0·25 c.c. of phenol reagent and 2·5 c.c. phenol reagent made up to 25 c.c. with distilled water. 50 mm. of tyrosine solution corresponded to 39·8 mm. of the protein solution, *i. e.* protein solution $= \frac{50}{39{\cdot}8} \times 0{\cdot}5$ c.c. of 0·02% tyrosine solution, *i. e.* $\frac{50}{39{\cdot}8} \times \frac{0{\cdot}5}{100} \times 0{\cdot}02$ gm. of tyrosine, and since the protein solution was made up to 10 c.c. and the tyrosine to 25 c.c. the protein solution is equivalent to $\frac{50}{39{\cdot}8} \times \frac{0{\cdot}5}{100} \times 0{\cdot}02 \times \frac{10}{25}$ gm. tyrosine; but 3·5 c.c. of the ammonium sulphate liquid was used for the determination, and this corresponds to $\frac{1}{1\frac{1}{2}} \times 3{\cdot}5$ c.c. of the original cerebro-spinal fluid, so that 100 c.c. of the fluid corresponds to $\frac{50}{39{\cdot}8} \times \frac{0{\cdot}5}{100} \times 0{\cdot}2 \times \frac{10}{25} \times \frac{100}{3{\cdot}5} \times \frac{1\frac{1}{2}}{1} = 0{\cdot}00216$ gm. tyrosine = pseudo-globulin + albumin in 100 c.c. of cerebro-spinal fluid.

Tube III.—Determination of albumin :

2·5 c.c. of cerebro-spinal fluid were taken and globulins precipitated by adding 2·5 c.c. saturated ammonium sulphate solution. It was centrifuged next day, 4·0 c.c. of clear liquid taken and precipitated twice as before, then dissolved in dilute alkali in 10 c.c. cylinder, and 0·1 c.c. of phenol reagent and 1·0 c.c. of 20% $NaCO_3$ added and made up to 10 c.c. and compared with "0·5" tyrosine standard (see above). 35 mm. of tyrosine solution corresponded to 43·5 mm. of protein solution, so that as before 100 c.c. of protein solution corresponded to $\frac{35}{43{\cdot}5}$ mm. $\times$ 0·02 gm. $\times \frac{0{\cdot}5}{100}$ c.c. $\times \frac{10}{25}$ c.c. $\times \frac{2}{4}$ c.c. $\times$ 100 = 0·00160 gm. of tyrosine = albumin in 100 c.c. spinal fluid.

Final Calculation.

Thus Tube I : albumin + pseudoglobulin + euglobulin in 100 c.c. fluid = 0·00270 gm. tyrosine.
Tube II : " " " " = 0·00216 gm. "
Tube III : " alone " " = 0·00160 gm. "
Therefore albumin = 0·00160 gm. tyrosine.
Pseudoglobulin . = 0·00216 − 0·00160 = 0·00056 gm. tyrosine.
Euglobulin . = 0·00270 − 0·00216 = 0·00054 gm. "
Therefore albumin content = 0·00160 × 27·5 = 0·044%
Pseudoglobulin " = 0·00056 × 26·2 = 0·015%
Euglobulin " = 0·00054 × 26·2 = 0·014%

Total = 0·073%

As it is not always convenient to perform an analysis on spinal fluids when they are fresh, and even in an ice-chest bacterial decomposition may occur, it is advisable to add the tungstic acid to one portion and the ammonium sulphate in appropriate amounts to two other portions, when the liquid may be kept at laboratory temperature.

SUMMARY.

1. A colorimetric method of determination of total protein content and that of the various protein fractions of cerebro-spinal fluids is described.

2. In normal fluid there is about 0·020% albumin and about 0·003% globulin, of which by far the greater part is pseudoglobulin.

3. In pathological fluids both albumin and globulin increase in quantity and generally the globulin more in proportion.

4. In 14 normal and pathological fluids the average ratio albumin : globulin was 6 : 1, in 3 tabetic fluids the ratio was 3 : 1, and in 16 general paretic fluids it was 1·3 : 1. The significance of these differences is discussed.

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